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# METHODS FOR THE DIRECT QUANTITATIVE DETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN URINE AND STOOLS.

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In the study of the inorganic metabolism of children it is frequently necessary to perform a large number of determinations of the various inorganic elements in urine and stools. The question of the amount of material available and the time required for a given determination becomes an important consideration. We have elsewhere reported simple methods for the quantitative estimation of sodium, potassium, calcium, and magnesium in serum (1, 2, and 3). It has been found possible to modify the sodium, potassium, and calcium methods so as to make them applicable to the acid extract of the partly ashed residue of urine and stools. For the determination of magnesium in urine and stools we have used the principle of alkalimetric titration of ammonium magnesium phosphate first suggested by Stolba (4). This procedure was subsequently modified by Mohr (5) and Kraus (6). Recently the same principle has been used by Bauzil (7), Angiolani (8), and Fiske (9) for the estimation of inorganic phosphates in urine. By the use of the methods described below a considerable saving in time and material is effected. The degree of accuracy of these methods is indicated in the tables.

## *Preparation of Material.*

*Stools.*—The fresh stool for a measured period is collected in a weighed porcelain dish. This is heated on the water bath until dry. 95 per cent alcohol is added and evaporated. This latter procedure is repeated, making two additions of alcohol in all. After thorough drying over the water bath the dish and contents

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are weighed and the weight of the air-dried stool is found. The fecal material is then ground to a fine powder and placed in a stoppered container. 2 gm. of this material are weighed in a platinum crucible and ashed for  $1\frac{1}{2}$  hours by the Stolte (10) method.<sup>1</sup> An ash-free filter paper (No. 40 Whatman, 11 cm. in diameter) is then washed by allowing 20 to 30 cc. of 0.5 N HCl to run through it. The platinum crucible containing the partly ashed stool is placed on the water bath and 10 cc. of 0.5 N HCl are added. After this has become hot it is transferred with a 10 cc. pipette and filtered through the washed filter paper into a 100 cc. flask. The procedure is repeated until the volume has been made up to 100 cc. The results obtained on this extract are identical with those found on a solution of completely ashed stool (Table VI).

*Urine.*—A measured quantity of urine (50 or 100 cc.) is evaporated in a platinum dish, ashed, and extracted in a similar manner to the stool. The extract is then made up to the original volume of the urine.

### *Sodium.*

The sodium is determined directly by precipitation as the pyroantimonate. As the precipitation must be carried out in an alkaline medium, calcium and any other elements present, which would form insoluble compounds such as tertiary calcium phosphate, must be removed, otherwise they would interfere with the gravimetric determination of the sodium pyroantimonate. This is accomplished by the following procedure. 15 to 20 cc. of the stool extract or 5 to 10 cc. of the urine extract are placed in a platinum dish and evaporated to dryness. The ash is transferred to a graduated centrifuge tube with 2.5 cc. of 0.5 N HCl. 3 cc. of a saturated solution of ammonium oxalate are added and the mixture is allowed to stand for 10 minutes. This precipitates practically all the calcium. Concentrated  $\text{NH}_4\text{OH}$  is then added to 7 cc., the contents are mixed and allowed to stand 45 minutes.

<sup>1</sup> The platinum dish which contains the material to be ashed is placed in a quartz dish 10 cm. in diameter and 6 cm. deep, in the bottom of which are placed several pieces of porcelain. The outer dish is gradually heated with a Meeker burner until no more fumes are given off when the flame is turned on full until the charred material is immobile. The large dish is then covered with a quartz plate and heating continued for  $1\frac{1}{2}$  hours.

Magnesium is thereby precipitated as ammonium magnesium phosphate. The sample is centrifuged for 5 minutes, after which 5 cc. of the supernatant fluid are placed in a platinum dish and evaporated to dryness. The dish is placed in the oven at 100°C. for a few minutes to thoroughly dry the residue in order to avoid spattering during the subsequent ashing. The sample is then ashed by the Stolte method for 15 to 30 minutes. This volatilizes all the ammonium salts. A small amount of ash remains in the dish. This dissolves readily in 2 cc. of 0.1 N HCl. A drop of phenolsulfonephthalein is added and the solution made just alkaline by the addition of 2 or 3 drops of 10 per cent KOH. This solution which contains all the sodium present in the original sample is now ready for the direct estimation of this element.

TABLE I.

*Determination of Sodium in Known Solutions of Inorganic Salts.\**

Amount of sample.	$\text{Na}_2\text{H}_2\text{Sb}_2\text{O}_7 + 6\text{H}_2\text{O}$ calculated.	$\text{Na}_2\text{H}_2\text{Sb}_2\text{O}_7 + 6\text{H}_2\text{O}$ found.	Error.
cc.	mg.	mg.	per cent
$\frac{3}{4}$ of 10	37.5	36.0	-4.2
$\frac{5}{8}$ " 10	37.5	38.1	+1.7
$\frac{5}{8}$ " 15	56.0	54.7	-2.3
$\frac{5}{8}$ " 15	56.0	56.2	+0.4
$\frac{5}{7}$ " 20	75.0	72.5	-3.3
$\frac{5}{8}$ " 20	75.0	72.8	-3.0

\* Composition of Solution Y.

NaCl.....	0.241 gm.
KCl.....	0.201 "
$\text{MgSO}_4 + 7 \text{H}_2\text{O}$ .....	0.130 "
$\text{Ca}_3(\text{PO}_4)_2$ .....	0.330 "
$\text{CaCO}_3$ .....	0.500 "
0.5 N HCl to.....	200 cc.

To the sample, prepared as outlined above, are added 10 cc. of the potassium pyroantimonate reagent followed by 3 cc. of 95 per cent alcohol. The alcohol should be added, drop by drop, and the specimen stirred with a rubber-tipped rod. After standing 30 minutes, the precipitate is transferred to a weighed Gooch crucible and washed with 5 to 10 cc. of 30 per cent alcohol. The crucible is dried at 110°C. for 1 hour,<sup>2</sup> cooled in a desiccator for 30 minutes,

<sup>2</sup> The temperature is gradually raised to 110°C.



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and weighed. The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

The method of preparation of the potassium pyroantimonate reagent has been fully described in a former paper on the determination of sodium in serum (1). The details of the method of preparation of the Gooch crucibles, the precautions to be observed during the addition of the alcohol and the filtration and also the care of the platinum are fully outlined in the same paper.

The results given in Table I show that as little as 3 or 4 mg. of sodium may be quantitatively recovered from solutions containing relatively large amounts of calcium phosphate.

### *Potassium.*

The potassium method is identical with that reported a short time ago by the authors for the estimation of this element in serum (2). The optimum amount of stool extract for the potassium determination is generally 1 cc. The concentration of this element in urine, however, is so high that it is necessary to take only 0.2 to 0.5 cc. The sample to be analyzed is placed in a graduated centrifuge tube and diluted with distilled water to 2 cc. The centrifuge tube should be previously cleaned with the use of a brush, washed out with strong cleaning fluid (commercial  $\text{H}_2\text{SO}_4$  and potassium dichromate), and then thoroughly rinsed with distilled water. If the tubes are not cleaned in this manner the precipitate will adhere to the sides and low results will be obtained. 1 cc. of the sodium cobalti-nitrite reagent is then slowly added, drop by drop. The contents of the tube are mixed and allowed to stand for  $\frac{1}{2}$  hour. The volume is made up to 5 cc. with water and the contents again mixed and the tube centrifuged for 7 minutes at about 1,300 revolutions per minute. The precipitate will then be found at the bottom of the tube. All but 0.2 to 0.3 cc. of the supernatant fluid is removed. This is accomplished by means of the following apparatus. Through one opening of a two-holed cork is inserted a glass tube by means of which a positive pressure can be made in the centrifuge tube. Through the other hole is placed a tube which reaches to about 3 or 4 mm. above the precipitate. The lower end of this tube is drawn out to a bore of about 1 mm. and curved so that the opening is directed upward. By

fitting the cork to the centrifuge tube and blowing through the first opening the supernatant fluid can be readily removed without disturbing the precipitate. 5 cc. of water are allowed to run down the side of the tube which is then gently agitated so that the added water is mixed thoroughly with the residual reagent. Care should be taken that the precipitate itself is disturbed as little as possible. This may be accomplished by holding the tube vertically and gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the supernatant fluid. The tube is then centrifuged for 5 minutes. The procedure is repeated three times so that the precipitate is washed four times in all. The supernatant fluid from the last washing should be perfectly clear. After the removal of the fluid from the final washing the precipitate is ready to be titrated.

*Titration.*—An excess of 0.02 N potassium permanganate (generally 2 to 5 cc.) is added to the precipitate followed by 1 cc. of 4 N  $\text{H}_2\text{SO}_4$ . It is rather difficult to judge the amount of permanganate necessary to be added, but by carefully watching the tube while it is being heated, more can be added. The precipitate is thoroughly mixed with the permanganate and  $\text{H}_2\text{SO}_4$  by means of a glass rod and the tube is placed in the boiling water bath. At the end of 20 to 25 seconds the tube is examined and if the pink color of the permanganate has nearly disappeared, more permanganate is added from the micro-burette. In this way it is not difficult to find out how much permanganate is necessary to constitute an excess. At the end of 1 minute from the time the heating is begun, the solution should be of a perfectly clear pink color. If all the precipitate is not oxidized, the contents will be cloudy and the color will be seen to fade. Heating should then be continued until the solution is clear and pink. When the heating is continued too long, the contents again become cloudy and of a brownish color. If this is allowed to happen, the sample must be discarded as high results will be obtained. 2 cc. of 0.01 N sodium oxalate are promptly added and the contents mixed. If this is not sufficient to decolorize the permanganate, another 2 cc. should be immediately added. The excess of oxalate is then titrated with 0.02 N potassium permanganate delivered from a micro-burette, graduated in 0.02 cc., until a definite pink color is obtained which lasts for 1 minute.

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*Calculation.*—1 cc. of 0.01 N potassium permanganate will oxidize a quantity of potassium cobalti-nitrite corresponding to 0.071 mg. of potassium. Thus, if 2 cc. of 0.02 N potassium permanganate are originally added and 0.43 cc. of the same solution used in the final titration and 2 cc. of 0.01 N sodium oxalate are required to decolorize the sample after the first oxidation, then  $2.43 - 0.03$  (the amount of permanganate necessary to colorize the same quantity of water)  $\times 2$  (to convert 0.02 to 0.01 N)  $- 2.00$  (cc. of 0.01 N sodium oxalate added to decolorize the sample)  $\times 0.071 = 0.199$  mg. of K in sample.

The details of the preparation of the reagents have been given in a former paper on the determination of potassium in serum.<sup>3</sup>

TABLE II.

*Determination of Potassium in Known Solutions of Inorganic Salts Containing an Excess of Calcium Phosphate.\**

Amount of sample.	K present.	K found.	Error.
cc.	mg.	mg.	per cent
0.4	0.215	0.218	+1.4
0.5	0.269	0.274	+1.8
0.7	0.376	0.384	+2.1
1.0	0.538	0.538	$\pm 0.0$

\* Composition of Solution C.

$\text{Ca}_3(\text{PO}_4)_2$ .....	2.5820 gm.
KCl.....	0.2052 "
NaCl.....	0.2063 "
$\text{MgSO}_4 + 7 \text{H}_2\text{O}$ .....	0.6100 "
N HCl to.....	200 cc.

The results of the estimation of potassium by this method in a known solution of inorganic salts are given in Table II. It should be noted that even a large amount of phosphate does not affect the potassium determination.

### *Calcium.*

The calcium method is identical with that reported a short time ago by the authors for the estimation of this element in serum (3).

<sup>3</sup> Kramer and Tisdall (2), p. 343.

The concentration of calcium in the stool extract, however, is so high that dilution is necessary. 5 cc. of the extract are diluted to 50 cc. with distilled water. The optimum amount of this solution for the calcium determination is generally between 1 and 4 cc. The amount of the urine extract corresponding to 1 to 4 cc. of urine is also found to be quite satisfactory.

The sample (generally 2 cc.) is measured into a graduated centrifuge tube previously cleaned with commercial  $\text{H}_2\text{SO}_4$  and dichromate and the volume made up to 3 or 4 cc. with distilled water. A drop of phenolsulfonephthalein is added and 10 per cent  $\text{NH}_4\text{OH}$  (10 cc. concentrated  $\text{NH}_4\text{OH}$  in 90 cc. of  $\text{H}_2\text{O}$ ) until the solution is alkaline. Approximately  $\text{N}$   $\text{H}_2\text{SO}_4$  is added until the solution is just acid and any phosphates that may have been precipitated are redissolved. 1 cc. of approximately  $\text{N}$  oxalic acid is added followed by 1 cc. of a filtered saturated solution of sodium acetate which should be added drop by drop. The contents are mixed and allowed to stand for  $\frac{3}{4}$  hour when they are centrifuged for 10 minutes at about 1,300 revolutions per minute. This throws all the calcium oxalate precipitate to the bottom of the tube. All but 0.3 cc. of the supernatant fluid is removed by means of the apparatus described under the potassium method. The remaining fluid and the precipitate are mixed by tapping the tube. Enough 2 per cent ammonia (2 cc. of concentrated ammonia diluted to 100 cc.) is then added to bring the volume to 4 cc., care being taken to wash the sides of the centrifuge tube free from adherent oxalic acid. The tube is then centrifuged for 5 minutes. This procedure is repeated twice, thus making three washings in all. After the third washing the supernatant fluid is removed, the tube is shaken to suspend the precipitate, 2 cc. of approximately  $\text{N}$  sulfuric acid are added, and the tube is warmed in the boiling water bath for a few minutes and titrated with 0.01  $\text{N}$  potassium permanganate until a definite pink color persists for at least 1 minute when viewed under a good light against a white background. The strength of the permanganate solution is determined by titrating against 0.01  $\text{N}$  sodium oxalate (Sörensen).

*Calculation.*—The number of cc. of 0.01  $\text{N}$  potassium permanganate used (generally 0.5 to 2 cc.)  $-$  0.02 cc. (the blank)  $\times$  0.2 = mg. of calcium in the sample.

*Preparation of Reagents.*—0.01  $\text{N}$  sodium oxalate (Sörensen) is

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the only reagent that must be quantitatively accurate. An 0.1 N sodium oxalate (Sørensen) is prepared in the usual way. 6.7 gm. of sodium oxalate (Sørensen) are dissolved in water. Solution is facilitated by the addition of 5 cc. of concentrated sulfuric acid and the volume made up to 1 liter. This is diluted ten times to make a 0.01 N solution. The former solution will keep indefinitely while the latter has been found still unchanged after the lapse of 2 months.

*Approximately N Oxalic Acid.*—This is prepared by dissolving 63 gm. of oxalic acid (Kahlbaum or J. T. Baker, c. p., calcium-free) in a liter of water. The acid need be weighed only roughly.

*Approximately N Sulfuric Acid.*—50 cc. of concentrated sulfuric acid (c. p.) are diluted with water to 1 liter.

TABLE III.

*Comparison of Calcium Determination on Solutions of Ash of Infants' Stools by McCrudden's Method and the Authors' Method.\**

Specimen.	McCrudden's method. Ca per 0.5 gm. stool.	Authors' method. Ca per 0.5 gm. stool.	Difference.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	31.17	30.09	−3.0
II	39.89	40.05	+0.4
III	35.11	34.50	−1.7
IV	53.16	51.18	−3.7

\* We are indebted to Dr. S. G. Ross for most of the calcium and magnesium determinations made by McCrudden's method.

*Saturated Sodium Acetate Solution.*—This solution is made by adding an excess of the salt to water and allowing it to stand over night. The supernatant fluid is then filtered. Sodium acetate (J. T. Baker, c. p.) does not contain calcium.

A comparison of the results obtained by this method and by the McCrudden method (11) on a solution of stool ash is given in Table III.

### *Magnesium.*

The principle used for the determination of magnesium is that originally advanced by Stolba (4) in 1877. The calcium is precipitated as the oxalate. The magnesium is then precipitated as

ammonium magnesium phosphate. An excess of HCl is added and the following reaction takes place:



The  $\text{H}_3\text{PO}_4$  is then titrated with 0.1 N NaOH to  $\text{NaH}_2\text{PO}_4$ , the pH of which is 4.4. The indicator used to detect the end-point is cochineal. This indicator changes from yellow to purple at pH 4.8. The error produced by titrating to this pH instead of pH 4.4 is small (9). 0.1 gm. molecules of  $\text{NH}_4\text{MgPO}_4$  when titrated with 0.3 gm. molecules of HCl yields 0.1 gm. molecules of  $\text{H}_3\text{PO}_4$ . When this is titrated back with 0.1 N NaOH to a pH of 4.8, one

TABLE IV.

*Determination of Magnesium in Samples of Solution A.\**

Solution A.	Titration reading. 0.1 N acid.	Magnesium found.	Magnesium present.	Error.
cc.	cc.	mg.	mg.	per cent
3	0.75	0.91	0.90	+1.1
5	1.20	1.45	1.50	-3.3
10	2.50	3.02	3.00	+0.7
20	5.10	6.17	6.00	+2.8

\* Composition of Solution A.

MgSO <sub>4</sub> containing Mg.....	0.030 gm.
CaCO <sub>3</sub> .....	1.154 "
Na <sub>2</sub> HPO <sub>4</sub> + 2 H <sub>2</sub> O.....	0.617 "
Concentrated HCl.....	5 cc.
H <sub>2</sub> O to.....	100 "

equivalent of H has been replaced by sodium, leaving two equivalents still united to PO<sub>4</sub> as  $\text{NaH}_2\text{PO}_4$ . Therefore, two equivalents of H are equal to two equivalents of Mg; i.e., 1 cc. of 0.1 N HCl = 1 cc. of 0.1 N Mg solution = 1.21 mg. of Mg.

The procedure is as follows: 25 to 50 cc. of the urine extract or 10 to 30 cc. of the stool extract are placed in a 100 cc. beaker. To this is added a drop of phenolsulfonephthalein and 10 per cent  $\text{NH}_4\text{OH}$  (10 cc. of concentrated  $\text{NH}_4\text{OH}$  in 90 cc. of  $\text{H}_2\text{O}$ ) until the solution is just alkaline. 4 N  $\text{H}_2\text{SO}_4$  is added until the solution is acid and all the phosphates are redissolved. 10 cc. of saturated ammonium oxalate are then added to the stool extract or 5 cc. to the urine extract, mixed, and allowed to stand for 15 minutes.

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This precipitates the calcium as the oxalate. 1 cc. of 10 per cent  $(\text{NH}_4)_2 \text{HPO}_4$  is added to insure an excess of phosphate followed by 5 cc. of concentrated  $\text{NH}_4\text{OH}$ . The mixture is thoroughly mixed, allowed to stand 1 hour, and then filtered through 9 cm. of

TABLE V.

*Comparison of Magnesium Determinations on Solutions of Ash of Infants' Stools by McCrudden's Method and the Authors' Method.*

Specimen.	McCrudden's method. Mg. in 0.5 gm. stool.	Authors' method. Mg. in 0.5 gm. stool.	Difference.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	2.84	2.88	+1.4
II	2.29	2.16	-5.7
III	2.78	2.70	-2.9
IV	3.55	3.54	-0.3

No. 40 Whatman filter paper. The precipitate is all transferred from the beaker by the use of a rubber-tipped rod and 10 per cent  $\text{NH}_4\text{OH}$ . The ammonia is then all removed from the filter paper by washing it four times with 30 per cent alcohol. The filter paper with the precipitate which includes the calcium oxalate

TABLE VI.

*Comparison of Calcium, Magnesium, Sodium, and Potassium Determinations on the Solutions of Stool Residues Which Were Ashed 1½ and 12 Hours.*

Specimen.	Inorganic element.	Ashed for 1½ hours.	Ashed for 12 hours.
		<i>gm. per 24 hrs.</i>	<i>mg. per 24 hrs.</i>
I	Ca	1.728	1.692
	Mg	0.115	0.115
	Na	0.514	0.510
	K	0.630	0.649
II	Ca	2.244	2.352
	Mg	0.104	0.108
	Na	0.035	0.037
	K	0.243	0.249
III	Ca	1.083	1.095
	Mg	0.081	0.084
	Na	0.146	0.145
	K	0.338	0.336

is transferred to a 100 cc. beaker, about 30 cc. of warm water are added, and the filter paper and precipitate mixed by the use of a glass rod. 3 drops of tincture of cochineal<sup>4</sup> are added and an excess of 0.1 N HCl (generally 5 cc.). After 5 minutes the mixture is titrated with 0.1 N NaOH delivered from a burette graduated in 0.05 cc. until the color changes from a light yellow to a purple. This end-point is very definite and a decided change in color is produced by 1 drop of 0.1 N NaOH. The presence of the filter paper and the large amount of calcium oxalate do not interfere with the interpretation of this end-point. The number of cc. of 0.1 N HCl added — the number of cc. of 0.1 N NaOH  $\times$  1.21 = the number of mg. of magnesium in the sample. An analysis of the magnesium content of a known solution of stool and urine salts is given in Table IV. A comparison of the results obtained by the above method and by McCrudden's method (11) is given in Table V.

Table VI shows that practically identical results are obtained on the acid extracts of partly ashed stools as on the acid solution of the completely ashed stools.

#### CONCLUSIONS.

1. Rapid methods for the determination of sodium, potassium calcium, and magnesium in urine and stools, including a direct method for the determination of sodium in the presence of large quantities of other salts, particularly calcium phosphate, have been described.

2. The determination of potassium by means of sodium cobaltinitrite reagent has been used for the estimation of small amounts of this element in urine and stools. The error by this method is generally within 1 or 2 per cent.

3. The determination of calcium and magnesium has been compared with the results obtained by the standard McCrudden method. The deviation from the standard method has generally been within 3 per cent.

4. By means of these methods a considerable saving of time is effected and all the so called fixed alkali elements may be determined quantitatively in 50 cc. of urine or 2 gm. of dry stool.

<sup>4</sup> The tincture is made by digesting 1 part of crushed cochineal with 10 parts of 25 per cent alcohol.



## 12 Inorganic Elements in Urine and Stools

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## A RAPID METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE.\*

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In making benzoate tests for renal efficiency we were confronted with the necessity for having a rapid and accurate method for the determination of hippuric acid in urine. The Folin-Flanders method which we have been using required more time than was thought necessary. By means of this method analyses could be made in 9 or 10 hours when necessary, but with the routine of teaching and other university work 24 hours were usually required. It was our object to devise a method which would conserve the accuracy of the Folin-Flanders (1) method, but one which could be completed within 2 or 3 hours and be as applicable for hospital routine work as are any of the other modern biochemical methods.

A careful review of the more recent methods for the determination of hippuric acid shows that at present there is only one method which fulfils the requirements of accuracy and simplicity. This is the method of Folin and Flanders. Two other methods which appeared at about the same time, Steenbock's (2) and Hryntschak's (3) methods meet the requirements of accuracy fairly well but are too tedious to compete with the Folin-Flanders method. Ito's (4) method appearing 4 years later is more complicated than those mentioned above and does not represent an advance in this field. Steenbock's and Hryntschak's procedures depend upon the isolation and weighing of benzoic acid, which are accompanied by slight losses, more in the latter method than in the former, and are necessary only in those cases in which benzoic acid cannot be directly titrated, as for instance, in the presence of other titratable

\*Acknowledgment is made to the Graduate School of the University of Minnesota for the purchase of a portion of the chemicals used in this work.

acids. Since there are no other acids present in the final extraction and titration stages of the Folin-Flanders method, titration in this case is not only easier to accomplish but more accurate.

Folin and Flanders proved that their method gave quantitative results with pure hippuric acid solutions which we have confirmed many times in the last few years. They did not compare their method, as applied to urine with any other procedure of analysis, nor as far as we can find, has any other investigator. They have assumed, however, that their method gives the most accurate results of any method devised up to that time. We have proved in the experiments which are directly to follow that the Folin-Flanders method does correctly estimate the amount of hippuric acid that can be extracted directly from urine by means of ethyl acetate.

*Experiment 1.*—0.561 gm. of pure sodium hippurate was dissolved in 100 cc. of water, 1 cc. of concentrated nitric acid added, and the mixture then extracted with ten 50 cc. portions of ethyl acetate, shaking exactly 2 minutes each time. The aqueous mixture left was then filtered, the filtrate evaporated to dryness over night on the steam bath with 10 cc. more of 5 per cent sodium hydroxide than that required for neutralization of the nitric acid present. The residue was then analyzed for any remaining hippuric acid by the Folin-Flanders method. The titration value was 0.07 cc., which is the ordinary blank of the method. The hippuric acid was completely extracted by this procedure.

100 cc. of urine, the hippuric acid titration value of which was 13.58 cc. of one-tenth normal sodium ethylate were acidified with 2 cc. of concentrated nitric acid and extracted with ten 50 cc. portions of ethyl acetate, shaking 2 minutes each time. The combined extracts were washed with two 200 cc. portions of the Folin-Flanders sodium chloride solution and then steam distilled until all of the ethyl acetate and approximately 300 cc. of water had passed over. The aqueous solution of hippuric acid remaining in the distilling flask was quantitatively transferred to a casserole and analyzed according to the Folin-Flanders method. The titration value was 13.43 cc. of one-tenth normal sodium ethylate, agreeing with the value obtained directly as well as duplicates can usually be obtained by this method.

#### *Experimental Methods of Analysis.*

Our problem resolved itself into increasing the speed of the hydrolysis of hippuric acid either by acids or alkalies and the effective oxidation of urinary pigments and other disturbing substances. Without going into the details of many experiments

carried out it may be stated that by using 15 gm. of solid sodium hydroxide in hydrolyzing the hippuric acid of 100 cc. of urine at the boiling point for 30 minutes and subsequently acidifying, extracting, and titrating, results were obtained that were, in one experiment, 22 per cent higher than the known titration value for this specimen of urine. It was also found that values from 10 to 33 per cent higher than those obtained by the Folin-Flanders method resulted when urine was boiled with an equal volume of a mixture of concentrated nitric and sulfuric acids for 30 minutes in a process that gave 100 per cent recovery when applied to solutions of pure hippuric acid. Oxidation of the urine with alkaline potassium permanganate after the plan of Hryntschak was tried and yielded such promising results that the details of one typical experiment are given below:

*Experiment 2.*—50 cc. of urine were boiled with 7.5 gm. of solid sodium hydroxide and 1.5 gm. of potassium permanganate for 30 minutes in a Kjeldahl flask with a rather closely fitting test-tube condenser in the neck. The flask was cooled and 50 cc. of concentrated nitric acid slowly poured down the side of the condenser. The brown mixture cleared up after boiling a few minutes, but this was continued for 30 minutes, then cooled and extracted as in the Folin-Flanders procedure using comparative amounts of the various materials; The titration value was 16.72 cc. of one-tenth normal sodium ethylate; by the regular Folin-Flanders method, 16.95 cc. In a series of 12 analyses made in this way it was found that values from 97 to 99 per cent of the Folin-Flanders figures could always be obtained when these were as large as 15 cc., but with lower values the error was sometimes as large as 25 per cent. This was believed to be due to the action of the potassium permanganate on the benzoic acid present as it was always most pronounced in the urines which were the most dilute and therefore containing less of the other substances to combine with the permanganate. It was difficult to estimate the correct amount of potassium permanganate to be added in each case and it frequently happened that 1.5 gm. were a greater amount than could be reduced beyond the manganate stage and 0.5 gm. portions of sodium bisulfite had to be added to complete the reduction. It was also found that if this method were applied to a pure solution of hippuric acid, allowing the potassium permanganate to act only 2 or 3 minutes before reducing it with sodium bisulfite that it was impossible to obtain more than 95 per cent of the theoretical amount. In Hryntschak's method the urine was boiled with 10 gm. of sodium hydroxide for 2.5 hours then 10 gm. of potassium permanganate were added and the boiling was continued for 6 or 7 minutes. The excess of permanganate was removed by adding about 15 gm. of sodium bisulfite prior to acidification and extraction. He subjected benzoic acid to the same conditions and was able to recover

98.24 and 98.17 per cent in two experiments and concluded from this that potassium permanganate did not destroy any benzoic acid. This is contrary to our findings using the more sensitive titration method.

We were reluctant about giving up the use of potassium permanganate because the subsequent chloroform extracts were always practically colorless and remained so until the definite pink end-point of titration was reached. No decidedly yellow extracts such as are rather frequent in the Folin-Flanders method were ever encountered. It was found by one of us that if a small quantity of magnesium oxide were present the effect of the permanganate in decreasing the titration value was prevented. The details of the method as we have adopted it follow:

### *Description of the Method.*

50 cc. of urine are treated with 7.5 gm. of sodium hydroxide and 0.5 gm. of magnesium oxide in a 500 or 800 cc. Kjeldahl flask. This mixture is boiled at such a rate as to bring its volume down to approximately 25 cc. in the course of half an hour. At the end of this time, while still at the boiling temperature, 1.0 cc. of a 7 per cent solution of potassium permanganate (a solution approximately saturated at room temperature) is added, care being taken to rinse down any that may remain on the neck of the flask with the smallest possible amount of water since no unchanged permanganate must be present when the acid is subsequently added. The flask with its brown contents is twirled gently for a minute or two, cooled under the tap, a fairly closely fitting test-tube condenser placed in the neck and 30 cc. of concentrated nitric acid slowly poured in down the side of the condenser. The mixture, which rapidly clears up on the addition of the acid, is now gently boiled for 45 minutes (30 minutes are sufficient for accurate results, but a less colored, more easily titratable extract is obtained by boiling it 45 minutes) with a good current of water flowing through the condenser, cooled under the tap, and the extraction with chloroform carried out approximately according to the Folin-Flanders method. The condenser is rinsed down with 25 cc. of water to remove any benzoic acid sublimed on the bottom of the condenser, the contents of the flask are transferred to a 500 cc. separatory funnel containing 25 gm. of solid ammonium sulfate. The flask is rinsed with 20 cc. of water which is poured into the separatory funnel. After dissolving the ammonium sulfate the benzoic acid is extracted successively with one 50 cc., one 35 cc.,

and two 25 cc. portions of neutral, well washed chloroform. The first 2 portions of chloroform are used to rinse the Kjeldahl flask. The combined extracts in a second separatory funnel are washed once with 100 cc. of the Folin-Flanders salt solution (containing 1.0 cc. of concentrated HCl in 2 liters of saturated NaCl solution) and drawn off through a dry filter paper into a dry Erlenmeyer flask. The separatory funnel from which the extract was drawn is rinsed with 20 cc. of chloroform. This is drawn off into a small beaker to which the wet filter paper had been transferred. The paper is rinsed with the chloroform and the latter is poured through a dry filter into the main bulk of extract in the Erlenmeyer flask. 4 drops of 1 per cent phenolphthalein in absolute alcohol are added and the benzoic acid solution titrated to a faint, but definite pink with tenth normal sodium ethylate. The preparation and standardization of this alkali solution are adequately described in the original paper of Folin and Flanders.

We have found that the following treatment of the chloroform used in this method insures a product that is reliable as far as its neutrality is concerned:

New chloroform, which is of the U. S. P. grade and contains about 0.75 per cent of ethyl alcohol, should be washed with an equal volume of distilled water twice before being used for the extraction of benzoic acid. Chloroform which has been used in analysis and therefore contains sodium benzoate and alcohol is first filtered through a dry filter paper which removes a considerable part of the sodium benzoate in those determinations in which the titration figure was fairly large. It is now washed successively with equal volumes of tap water, once; tap water containing 5 to 10 cc. of a saturated solution of NaOH, twice; tap water, twice; and distilled water, once; six washings in all. Since the accuracy of this method depends primarily upon the use of a sample of chloroform which not only reacts neutral when tested, but which must remain neutral after being shaken with nitric acid, we have used the test which follows to determine this point:

155 cc. of chloroform, the amount used in an analysis, washed as described above, are shaken with dilute nitric acid, washed with 100 cc. of the Folin-Flanders salt solution, filtered through a dry paper, and titrated. The titration of this amount of chloroform suitable for use should not exceed 0.10 cc. of tenth normal sodium ethylate.

The application of this method or that of Folin and Flanders requires the removal of protein from the urine when this is present, as in nephritic urines. Directions for doing this have already been published, but perhaps may be repeated here.

The albuminous urine is collected in 2 per cent nitric acid which was found by Raiziss and Dubin (5) to be effective in preventing the hydrolysis of hippuric acid. 15 cc. of this dilute nitric acid are sufficient for a 3 hour nephritic urine. 50 cc. of this urine, treated with 3 or 4 drops of 0.1 per cent methyl red solution in alcohol, are brought to the first definite yellow by the addition of approximately normal NaOH. The solution is then boiled, and during the boiling sufficient one-tenth normal HCl is added to produce the first definite red color. This procedure removes the albumin nearly quantitatively so that there is no increase in the resulting titration, as has already been shown (6). The coagulum of albumin on the filter paper is washed twice with 50 cc. of boiling water. The combined washings and main bulk of filtrate are evaporated rapidly over a free flame in an 800 cc. Kjeldahl flask after being made slightly alkaline to methyl red by the addition of a small amount of dilute alkali. Bumping and frothing, should they occur, are checked by adding a glass pearl and a drop of caprylic alcohol. By supporting the funnel in the neck of the flask by means of a slice of a large cork stopper the filtration and evaporation are continued simultaneously. When the contents of the flask have been evaporated to approximately 50 cc., 7.5 gm. of NaOH and 0.5 gm. of MgO are added and the analysis made according to the directions already given.

In Table I are given the comparative results with various specimens of urine, normal and pathological, obtained by the new method and by that of Folin and Flanders. In a series of approximately half of the determinations one of us used one method and the other, the other method. The results of neither of us were known to the other until all the determinations of this series had been made, when they were compared. No. 19 in Table I is a comparison of the two methods with 50 cc. aliquots of a pure sodium hippurate solution. The only modification in this case was the reduction of the permanganate with 0.5 gm. of sodium bisulfite as a substitute for the urinary constituents which ordinarily function in this manner, prior to the acid treatment. It is noted that the agreement

between the two methods is good, as close in general as duplicates can be made by the older method, and that duplicates by the new method, where they have been made show a very close agreement.

TABLE I.

Urine No.	0.1 N Na ethylate.	
	New method.	Folin-Flanders method.
	<i>cc.</i>	<i>cc.</i>
1	4.50	4.70
	4.35	4.50
	4.80	
	4.50	
2	29.40	28.95
	29.50	29.60
3	13.95	13.55
		13.70
4	33.65	33.10
	33.50	33.80
5	1.05	0.95
6 P.*	24.55	24.80
7 P.*	26.60	26.75
8 P.*	24.50	24.50
9	14.20	14.20
10	20.10	19.80
11	8.50	8.65
12	5.25	5.35
13	8.65	8.70
14	17.55	16.95
15	8.55	8.80
16	8.95	8.80
17	12.56	12.35
18	13.75	14.00
	13.55	
19†	14.25	14.25
		13.80
		14.15

\* Urines designated by "P." are pathological specimens. All others are normal.

† 50 cc. of a sodium hippurate solution were used.

A few duplicate determinations have been made several days apart with no evidence of loss of hippuric acid in acid urines at room temperature preserved with a small amount of a 10 per cent solution of thymol in chloroform.



## CONCLUSION.

An accurate, rapid method for the determination of hippuric acid in urine is described which requires about 2 hours for completion with normal urine and about 3 hours with urine containing albumin.

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## NOTE ON A POSSIBLE SOURCE OF ERROR IN TESTING FOR BENCE-JONES PROTEIN.

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In a long series of attempts to produce Bence-Jones proteinuria in dogs by administration of agents known to cause lesions of the bone marrow, we found a possible source of error in making qualitative tests for this protein to which it may be well to call attention. If urine, especially of dogs, containing a small amount of serum protein is allowed to stand at room temperature for from 8 to 24 hours after voiding, it will occasionally be found that the heat coagulation test, at first clear, will after standing become less marked or even disappear entirely. On the other hand a considerable cloud will still be produced by potassium ferrocyanide and acetic acid; and the addition of an equal volume of a saturated solution of ammonium sulfate will also give a precipitate. This change occurs even when the urine is preserved with toluene, and is apparently due to the proteolytic enzyme of the urine. Now since it is well known that certain digestion products give the same heat precipitation and resolution reactions as Bence-Jones protein, the desirability of using fresh urine when testing for this substance is obvious. In working with dogs it is also necessary to collect the urine by catheter, or at least to watch the dog carefully so as to be certain of obtaining urine free from contamination (vomitus, feces). Even slightly contaminated urine from the cage was several times found to contain enough digestion products to give Bence-Jones reactions, which could not be confirmed in specially collected urine.

The use of toluene as a preservative for urine may temporarily give rise to confusion. If a couple of drops of toluene are emulsified by shaking in a test-tube with urine or water an opalescent

fluid results. If this be heated it becomes clear, and when cooled the cloudiness immediately returns, and this can be repeated, thus simulating the behavior of Bence-Jones protein. Either the emulsion becomes invisible when hot on account of the change of the refractive index of the toluene, or the latter goes into solution at boiling heat and is reprecipitated upon cooling. Very little agitation, *e. g.* filtration, of urine preserved with toluene can effect sufficient emulsification to cause this phenomenon; however, by confirmatory tests it is easily differentiated.

## ACERIN.

### THE GLOBULIN OF THE MAPLE SEED (ACER SACCHARINUM).\*

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#### INTRODUCTION.

In an earlier paper from this laboratory<sup>1</sup> it was indicated that the cotyledons of maple seed contained a high percentage of nitrogen, and preliminary experiments showed that a large amount of this nitrogen was present in the form of a globulin which could be extracted with water or with dilute solutions of sodium chloride. In the pure state the globulin is insoluble in water but owing to the presence of soluble salts in the seed it is extracted by small quantities of distilled water just as readily as by dilute solutions of sodium chloride. On diluting such aqueous extracts a large part of the globulin is precipitated.

The amount of total and soluble nitrogen in the cotyledons is shown in Table I.

The nitrogen in the 70 per cent alcoholic extract was non-protein in character, but the nature of the nitrogen compounds insoluble in dilute sodium chloride solution was not determined.

The amount of pure globulin obtained from 100 gm. of powdered seed was only about 6.5 gm. This corresponds to about only one-half of the amount of nitrogen soluble in a dilute solution of sodium chloride. However, no effort was made to secure a quantitative yield.

\* Read at the Chicago meeting of the American Association of Biological Chemists, December, 1920.

<sup>1</sup> Anderson, R. J., *J. Biol. Chem.*, 1918, xxxiv, 509.

The globulin separates on dialysis into small uniform globular particles which show no crystalline structure and it is precipitated completely from saline solutions by 0.6 saturation with ammonium sulfate. It has been isolated and purified by alternately precipitating it with ammonium sulfate and by dialyzing its dilute salt solutions. After dehydrating with alcohol and drying in vacuum it was obtained as a compact light gray or nearly white powder which was non-hygroscopic. Since this is the first protein obtained from maple seed we propose, provisionally, to call this globulin *acerin*.

TABLE I.  
*Nitrogen in Maple Seed.*

	<i>per cent</i>
Total nitrogen.....	4.40
Nitrogen soluble in 70 per cent alcohol.....	0.39
“ “ “ 5 “ “ sodium chloride.....	2.06
“ remaining in seed residue.....	1.93

TABLE II.  
*Composition of Acerin and Arachin.*

Constituents.	Acerin.	Arachin.
	<i>per cent</i>	<i>per cent</i>
C.....	51.44	52.15
H.....	6.80	6.93
N.....	18.34	18.29
S.....	0.55	0.40
O.....	22.87	22.23

A number of different preparations were made in different ways and on analysis, all gave practically identical results. These various preparations were so nearly ash-free that a non-weighable residue remained after combustion.

The percentage composition of acerin is very similar to that of legumin, amandin, or the globulin from cottonseed described by Osborne and Campbell<sup>2</sup> and Osborne and Voorhees.<sup>3</sup> However,

<sup>2</sup> Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1896, xviii, 609; 1898, xx, 348.

<sup>3</sup> Osborne, T. B., and Voorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 778.

the nitrogen distribution and particularly the percentage of basic amino-acids vary widely from results obtained by the above authors, indicating a decided difference of the molecular constitution of these proteins. Arachin, the globulin from peanut, recently described by Johns and Jones<sup>4</sup> is very similar in composition and nitrogen distribution to acerin, but the percentage of the basic amino-acids differs in the two globulins. These relations are indicated in Tables II and III.

TABLE III.  
*Nitrogen Distribution in Acerin and Arachin.*

Form of nitrogen.	Acerin.	Arachin.
	<i>per cent</i>	<i>per cent</i>
Amide nitrogen.....	2.53	2.03
Humin " .....	0.15	0.22
Basic " .....	4.86	4.96
Non-basic " .....	10.63	11.07
Basic nitrogen by Van Slyke method.		
Cystine.....	0.55	0.85
Histidine.....	1.43	1.88
Arginine.....	10.07	13.51
Lysine.....	6.07	4.98

#### EXPERIMENTAL.

The air-dried cotyledons, freed from the testa or outer brown membrane, were powdered and extracted with ether at room temperature. After the ether had evaporated, the seed residue was used for the isolation of the globulin.

A solution of the globulin is obtained on digesting this powdered material in a small quantity of water or in a dilute solution of sodium chloride. On filtering the extract through a layer of paper pulp a clear brownish yellow solution is obtained.

After extracting the powdered maple seed with 5 per cent sodium chloride solution and filtering as indicated above, the filtrate gave the following reactions: (1) It was slightly acid to litmus. (2) The globulin was precipitated on the addition of

<sup>4</sup> Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916-17, xxviii, 77.

dilute acids, but saturating the solution with carbon dioxide did not produce any precipitate. (3) The solution turned slightly cloudy when gradually heated to 50°C. The cloudiness increased with a rise in temperature and at 75°C. a flocculent precipitate began to form and at 100°C. the amount of the precipitate increased. (4) Ammonium sulfate added to the clear solution caused only a very faint cloudiness up to 0.2 saturation, but with 0.3 and 0.4 saturation, a heavy precipitate is produced. When this precipitate is filtered off further addition of ammonium sulfate up to complete saturation produces only a very slight cloudiness.

#### *Isolation of Acerin.*

Of the powdered ether-extracted seed, 200 gm. were digested in 900 cc. of 10 per cent sodium chloride solution to which were added 40 cc. of a saturated solution of barium hydrate. The amount of barium hydrate necessary to maintain a neutral reaction in the extract was determined by titration. The mixture was stirred for about 15 minutes and it was then filtered through a layer of paper pulp.

A perfectly clear brownish yellow filtrate was obtained. It was saturated with ammonium sulfate and the precipitate which formed was separated from the mother liquor as thoroughly as possible by centrifuging. The precipitate was dissolved by adding about 100 cc. of water and the solution was filtered through paper pulp. The globulin was again precipitated by saturating the filtrate with ammonium sulfate. The mixture was centrifuged; the precipitate dissolved in 100 cc. of water, filtered through paper pulp, and reprecipitated a third time with ammonium sulfate. After centrifuging, dissolving in water, and filtering through a layer of paper pulp, toluene was added and the solution dialyzed in a collodion bag suspended in distilled water. The water was frequently changed and the dialysis was continued until the solution was free from sulfates. The protein separated in the form of small uniform globular particles which showed no crystalline structure.

The precipitated globulin was removed from the dialyzer and collected on a Buchner funnel and washed with water. It was then suspended in 95 per cent alcohol, again filtered, and washed successively with 95 per cent alcohol, absolute alcohol, and ether,

and finally dried in vacuum over sulfuric acid. The dry substance was a heavy, nearly white powder, and it weighed 16.3 gm.

The substance was moistened with a little saturated solution of ammonium sulfate and dissolved by adding about 100 cc. of water. After filtering off a small amount of insoluble material through a layer of paper pulp the filtrate was again dialyzed. The precipitated globulin was dehydrated by treating it successively with 25, 50, 75, and 95 per cent alcohol and finally with absolute alcohol. It was filtered, washed with absolute alcohol and ether, and dried in vacuum over sulfuric acid. The dry substance weighed 13.5 gm. The substance was analyzed after drying at 110° in vacuum over phosphorus pentoxide.

0.1252 gm. substance lost 0.0067 gm. = 5.25 per cent  $H_2O$ .

0.1185 " " gave 0.0708 "  $H_2O$  and 0.2231 gm.  $CO_2$ .

0.7932 " " " 0.0351 "  $BaSO_4$ .

0.1420 " " required 18.5 cc. 0.1 N  $H_2SO_4$  (Kjeldahl).

Found: C, 51.22; H, 6.68; S, 0.60; N, 18.24 per cent.

### *Second Preparation of Acerin.*

#### *Extraction with Water.*

50 gm. of the powdered, ether-extracted maple seed were digested in 200 cc. of distilled water for about 5 minutes. The extract was then filtered through paper pulp and washed with water until 200 cc. of filtrate were obtained. Ammonium sulfate was added nearly to saturation; the precipitate was centrifuged, dissolved by adding 100 cc. of water, and the solution was filtered through paper pulp and the filtrate dialyzed under toluene until free from sulfate. The precipitated globulin was dehydrated and washed with alcohol and ether as before and dried in vacuum over sulfuric acid. The yield was 3.6 gm.

There is a sufficient amount of soluble salts in the maple seed to permit practically all of the globulin to be extracted with distilled water. The seed residue in the above preparation was extracted with 200 cc. of 10 per cent sodium chloride solution, but the extract contained an exceedingly small quantity of protein.

The globulin obtained above was identical in appearance and properties with the first preparation. It was analyzed without further purification after drying at 110° in vacuum over phosphorus pentoxide.



0.1652 gm. substance lost 0.0097 gm. = 5.87 per cent  $H_2O$ .  
 0.1555 " " gave 0.0944 "  $H_2O$  and 0.2947 gm.  $CO_2$ .  
 0.8454 " " " 0.0326 "  $BaSO_4$ .  
 0.1412 " " required 18.5 cc. 0.1 N  $H_2SO_4$  (Kjeldahl).  
 Found: C, 51.68; H, 6.79; S, 0.56; N, 18.34 per cent.

### *Third Preparation of Acerin.*

200 gm. of the powdered maple seed were digested in 800 cc. of distilled water for 15 minutes, filtered, and washed with water until 800 cc. were obtained. The filtrate was saturated with ammonium sulfate and the precipitate centrifuged and then transferred to a Buchner funnel and washed with saturated ammonium sulfate solution. The precipitated globulin was redissolved by adding 250 cc. of water, the solution filtered and dialyzed until free from sulfates. The contents of the dialyzer were transferred to a beaker and allowed to settle. The supernatant liquid was poured off and the globulin brought on a Buchner funnel and washed with water. It was then suspended in about 200 cc. of water and dissolved by adding about 5 per cent of sodium chloride. The solution was filtered and dialyzed until free from chlorides. The precipitated globulin was removed from the dialyzer, dehydrated with alcohol, washed in alcohol and ether, and dried in vacuum over phosphorus pentoxide. The dry substance weighed 12.6 gm. This preparation was analyzed after drying at  $110^\circ$  in vacuum over phosphorus pentoxide.

0.1285 gm. substance lost 0.0077 gm. = 5.99 per cent  $H_2O$ .  
 0.1208 " " gave 0.0751 "  $H_2O$  and 0.2279 gm.  $CO_2$ .  
 0.7884 " " " 0.0300 "  $BaSO_4$ .  
 0.1411 " " required 18.8 cc. 0.1 N  $H_2SO_4$  (Kjeldahl).  
 Found: C, 51.45; H, 6.95; S, 0.52; N, 18.65 per cent.

### *Fourth Preparation of Acerin.*

50 gm. of the powdered maple seed were digested in 200 cc. of 70 per cent alcohol for 2 hours with frequent shaking. It was then filtered on a Buchner funnel and washed with 70 per cent alcohol until the filtrate came through colorless. The seed residue was dried in vacuum over sulfuric acid and then digested in 200 cc. of 5 per cent sodium chloride solution to which sufficient barium hydrate had been added to maintain a neutral reaction

in the extract. It was filtered through paper pulp and washed with water until 300 cc. of extract were obtained.

The extract was precipitated by adding ammonium sulfate to 0.6 saturation. The mixture was then centrifuged and the globulin transferred to a Buchner funnel and washed with ammonium sulfate solution of the same strength. The precipitate was brought into solution by adding 100 cc. of water. It was again filtered through paper pulp and dialyzed until free from sulfate. The precipitated globulin was suspended in 100 cc. of water and dissolved by adding a little ammonium sulfate. The solution was filtered and again dialyzed until free from sulfates. The globulin which separated was dehydrated with 30, 50, 70, and 95 per cent alcohol and finally with absolute alcohol and ether and dried in vacuum over phosphorus pentoxide. The nearly white powder weighed 3.3 gm.

The extraction with 70 per cent alcohol removed about 16 per cent of solid matter from the maple seed but this material contained only 0.3 per cent of non-protein nitrogen.

After precipitating the globulin from the extract obtained on digesting the seed residue in 5 per cent sodium chloride with ammonium sulfate to 0.6 saturation, further addition of ammonium sulfate to the filtered extract gave no additional precipitate, showing that all of the protein was precipitated with the above concentration of ammonium sulfate. The globulin was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1553 gm. substance lost 0.0097 gm. = 6.24 per cent  $H_2O$ .

0.1456 " " gave 0.0883 "  $H_2O$  and 0.2748 gm.  $CO_2$ .

0.7749 " " " 0.0314 "  $BaSO_4$ .

0.1419 " " required 18.5 cc. 0.1 N  $H_2SO_4$  (Kjeldahl).

Found: C, 51.46; H, 6.77; S, 0.55; N, 18.35 per cent.

#### *Fifth Preparation of Acerin.*

The seed residue, after extracting 300 gm. of maple seed with 70 per cent alcohol, was digested in 1,500 cc. of 5 per cent sodium chloride solution containing 60 cc. of Baryta water. The extract was filtered and washed with 5 per cent sodium chloride solution until about 1,600 cc. of filtrate were obtained. The clear filtrate was precipitated by adding ammonium sulfate to 0.6 saturation. After centrifuging, filtering, and washing with ammonium sulfate

solution, the globulin was dissolved by adding about 300 cc. of water. This solution was filtered and dialyzed. The globulin was removed from the dialyzer and suspended in water and dissolved by adding a small amount of ammonium sulfate. After filtering the solution it was precipitated with ammonium sulfate to 0.6 saturation, filtered, washed with ammonium sulfate solution, dissolved in about 300 cc. of water, and dialyzed until free from sulfate. After dehydrating with alcohol and ether as before it was dried in vacuum over sulfuric acid. The yield was 19.5 gm. The substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1578 gm. substance lost 0.0097 gm. = 5.89 per cent H<sub>2</sub>O.  
 0.1485 " " gave 0.0913 " H<sub>2</sub>O and 0.2801 gm. CO<sub>2</sub>.  
 0.8011 " " " 0.0309 " BaSO<sub>4</sub>.  
 0.1412 " " required 18.4 cc. 0.1 N H<sub>2</sub>SO<sub>4</sub> (Kjeldahl).  
 Found: C, 51.44; H, 6.87; S, 0.53; N, 18.24 per cent.

#### *Sixth Preparation of Acerin.*

This was prepared from 300 gm. of powdered maple seed exactly as described for the fifth preparation. The dry product weighed 20 gm. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.2738 gm. substance lost 0.0160 gm. = 5.84 per cent H<sub>2</sub>O:  
 0.2578 " " gave 0.1556 " H<sub>2</sub>O and 0.4861 gm. CO<sub>2</sub>.  
 0.7552 " " " 0.0319 " Ba SO<sub>4</sub>.  
 0.1413 " " required 18.4 cc. 0.1 N H<sub>2</sub>SO<sub>4</sub> (Kjeldahl).  
 Found: C, 51.41; H, 6.72; S, 0.58; N, 18.23 per cent.

TABLE IV.

#### *Summary of Analyses of Acerin.*

Preparation.....	1	2	3	4	5	6	Average composition.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	51.22	51.68	51.45	51.46	51.44	51.41	51.44
H.....	6.68	6.79	6.95	6.77	6.87	6.72	6.80
S.....	0.60	0.56	0.52	0.55	0.53	0.58	0.55
N.....	18.24	18.34	18.65	18.35	*18.24	18.23	18.34
O (by difference)...	23.26	22.63	22.43	22.87	22.92	23.01	22.87

A composite sample containing all of the above preparations gave 0.55 per cent. of sulfur and 18.32 per cent of nitrogen (Table IV), which values are identical with the above averages.

After hydrolysis according to the method of Van Slyke<sup>5</sup> the nitrogen constituents were determined as shown in Table V.

The basic amino-acids were determined in the phosphotungstic acid precipitate by the micro method of Van Slyke.<sup>6</sup> The results calculated to the basis of the original globulin are given in Table VI.

TABLE V.  
*Nitrogen Distribution in Acerin.*

Form of nitrogen.	Amount.
	<i>per cent</i>
Amide nitrogen.....	2.53
Humin " .....	0.15
Basic " .....	4.86
Non-basic " .....	10.63
Total nitrogen recovered.....	18.17

TABLE VI.  
*Basic Amino-Acids in Acerin.*

	<i>per cent</i>
Cystine.....	0.55
Arginine.....	10.07
Histidine.....	1.43
Lysine.....	6.07

#### SUMMARY.

The principal protein of the seed of the silver maple (*Acer Saccharinum*) has been isolated and purified. This protein, for which we propose the name *acerin*, is a globulin. It could not be obtained in crystalline form but separated on dialysis in small uniform globular particles. The purified acerin is a nearly white heavy powder which on combustion leaves no weighable ash. A number of different preparations were made and all of these preparations showed close agreement on analysis.

<sup>5</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.

The average composition of acerin is as follows:

C, 51.44; H, 6.80; N, 18.34; S, 0.55; O, 22.87 per cent.

When analyzed by the Van Slyke method it was found that a considerable percentage of the basic nitrogen was present as lysine.

## DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

### I. THE COMPARATIVE INFLUENCE OF GREEN AND DRIED PLANT TISSUE, CABBAGE, ORANGE JUICE, AND COD LIVER OIL ON CALCIUM ASSIMILATION.\*

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The problem of the dietary factors influencing calcium assimilation in domestic animals is not new. In 1913 we drew attention to an observation (1), which, when worked out in its details will, no doubt, have a very important bearing on the question of calcium assimilation. In this early publication data were presented which showed that there were marked differences in the amount of calcium eliminated in the feces of a milking goat when that animal was changed from old dried roughage to green pasture, and after a period of fresh green grass intake placed in the metabolism cage and returned to the dried feed ration for a calcium balance experiment. After the period of green pasture feeding, the fecal calcium elimination was so reduced as to give a positive calcium balance as compared with a high fecal calcium output and negative calcium balance on the old dry roughage. Evidently something had been ingested with the green material that allowed a more perfect skeletal storage of calcium or a more complete assimilation of this element from the intestine.

In an earlier piece of work (2) we had observed that a negative calcium balance could prevail with a lactating cow for a very long time with a slow shrinkage in milk flow but no observable physiological disturbances. This animal was under actual quanti-

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tative observation for 110 days and on a ration consisting of ordinary oat straw, wheat bran (natural or washed), rice meal, and wheat gluten. The fecal output alone of calcium was nearly equal to the daily intake of this element. Her daily output of calcium oxide was approximately 50 gm. per day with an intake of but 25 gm. The constancy in maintaining the percentage of calcium oxide in the milk was remarkable. We would not want to leave the impression that there were not deep seated and seriously abnormal conditions developing in this individual and that ultimately nutritional failure and milk production must cease, but we did not observe this in a period of 110 days. We have observed (3) disaster in reproduction with cows where probably negative calcium balances are long continued.

In this same direction Forbes and his associates (4) have made important contributions showing that high milking cows receiving rations that are supposed to provide an ample intake of calcium, nevertheless may eliminate a larger amount of calcium than is ingested. Even the addition of calcium salts to a ration of grains, dry alfalfa hay, and corn silage did not lead in their experience to the establishment of positive calcium balances.

In a similar direction Meigs, Blatherwick, and Cary (5) have contributed interesting data showing that a dry but pregnant cow is probably not assimilating sufficient calcium from a calcium-rich ration such as dry alfalfa hay, corn silage, and a grain mixture for a positive balance, but is actually transferring calcium salts from her skeleton for fetal skeleton building. Meigs and his associates are inclined to interpret these observed negative calcium balances as only temporary and merely incident to the collection of the excreta and due to nervous disturbance of the animal. While we recognize the possibility of such a factor as operative with some individuals, yet we believe that the main factor of influence in this connection is of dietary origin.

While the problem of calcium assimilation and metabolism is of very great importance in relation to growth, milk production, and egg production of our farm animals, it is of equal importance in human nutrition, and no doubt likewise related to dietary factors other than mere calcium supply. In a recent short note (6) where we briefly discussed this subject we said:

" . . . In the case of nursing women the relation of diet to a positive or negative calcium balance and to dental conditions will assume new aspects.

"The supposition that we are dealing with something influencing calcium assimilation and which is more abundant in green than in dried plant tissue and consequently variable with the season's milk, would explain the variations in the seasonal frequency of rickets, as observed and commented upon by Hess (7)."

In continuation of this line of reasoning we are assuming that it is entirely probable that the factor or factors shown to be operative in optimum calcium assimilation in any one of our farm animals can be translated as applicable not only to other types of animals but to human nutrition as well. No doubt there will be species differences. One species, under adverse conditions will assimilate calcium more completely than another, but these differences will be quantitative and not qualitative. Just as the guinea pig is more sensitive to a lower supply of the antiscorbutic vitamine than the rat, cow, or pig, so the human infant and puppy are probably more sensitive than the rat or pig to a low supply of the dietary factors affecting calcium assimilation, but it is "a difference of degree and not of kind."

The statement that faulty calcium assimilation or poor bone formation is due to lack of a balance of dietary factors is too indefinite to satisfy students of nutrition. Of course, it is self-evident that a low calcium- or a low phosphorus-containing ration would be a primary factor in poor skeletal development with a rapidly growing species, but the real problem before us is the disclosure of the nature of that dietary factor whose absence leads to faulty calcium assimilation even in the presence of an ample supply of this element.

Faulty calcium assimilation extending over a comparatively long time can occur in cows and mature swine without an exhibition of the quick and complete collapse shown by a growing puppy suffering from rickets, and yet we have no doubt that some of the nutritive failures exhibited by growing swine (8) and even mature swine and mature cattle on certain restricted diets will ultimately be classed as in the main a condition simulating rickets.

In this paper there will be presented only what preliminary data we have accumulated on the influence of dietary factors on calcium assimilation by the dry and milking goat. Work with other



types of animals and with food materials other than those used in these experiments is now in progress and will be reported on when the accumulated data warrant it.

#### EXPERIMENTAL.

The goats used were common American grades with no distinct breeding. They were confined in our metabolism cages with quantitative collection of the excreta. When milking animals were under observation they were milked twice daily. Analyses were applied to the weekly collection of feces and to the weekly composites of aliquots of urine and milk taken daily. Calcium determinations were made on the feeds, milk, and feces by the McCrudden method, after ashing. In the urine the calcium estimation was made directly and without ashing as further suggested by McCrudden (9).

#### *Record of Animal 1.*

Animal 1 was a milch goat producing 700 to 800 cc. of milk per day. She, as well as the others, was fed a grain mixture consisting of 30 parts of yellow corn, 15 of oil meal, 30 of whole oats, 24 of wheat bran, and 1 of common salt. The roughages were varied in the successive periods of observation being, respectively, alfalfa hay, oat straw, green oats, and dried green oats. In view of the fact that Forbes, Meigs, and their associates, as already stated, observed a negative calcium balance in feeding cows with alfalfa hay—a roughage distinctly high in its calcium content—we used alfalfa hay in the first period. This gave us data for comparing the behavior of the cow with the goat.

*Alfalfa Hay Period.*—After 2 weeks preliminary feeding, collections were made for a period of 5 weeks on the alfalfa ration. During this time 3,500 gm. of alfalfa and 3,500 gm. of grain mixture were consumed weekly. Contrary to expectations the animal did not go into negative calcium balance as can be seen in Table I where the record of this as well as of the other trials are tabulated. As this animal was purchased locally and had been receiving a varied and unknown ration before her purchase there is a possibility that previous storage of the factor influencing calcium assimilation had occurred which later during the alfalfa feeding period aided in maintaining normal calcium assimilation. We are in-

clined to doubt the validity of this assumption, in the first place, because the record was started in April—a time of the year when the animal kept on ordinary rations would be expected to be depleted of this dietary factor—and in the second place, because on the oat straw ration, immediately following, a negative calcium balance was readily established. It seems plausible to assume that

TABLE I.

*Record of the Calcium Balance of Animal 1.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Alfalfa hay period.								
	<i>gm.</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Apr. 18-25.....	2,330	2.74	63.84	0.32	12.31	86.45	76.47	+ 9.98
“ 25-May 2.....	2,240	3.01	67.42	0.18	13.72	86.45	81.32	+ 5.13
May 2-9.....	2,341	3.22	75.49	0.29	12.50	86.45	88.28	- 1.83
“ 9-16.....	2,248	3.07	69.09	0.17	15.61	86.45	84.87	+ 1.57
“ 16-23.....	2,375	2.87	68.16	0.09	11.70	86.45	79.95	+ 6.50
Oat straw period..								
June 6-13.....	1,363	1.84	25.18	0.02	10.48	12.92	35.68	-22.76
“ 13-20.....	1,222	1.70	20.74	0.10	7.39	8.69	28.23	-19.54
“ 20-27.....	1,128	1.70	19.74	0.02	8.13	8.71	27.89	-19.18
“ 27-July 4....	1,035	1.29	13.37	0.02	7.38	8.50	20.77	-12.27
Green oats period.								
July 4-11.....	888	0.68	6.06	0.02	6.20	8.04	12.28	- 4.24
“ 11-18.....	871	0.65	5.66	0.10	6.63	8.00	12.39	- 4.39
“ 18-25.....	761	1.07	8.15	0.07	6.21	7.78	14.43	- 6.65
“ 25-Aug. 1....	811	1.20	9.66	0.10	5.51	8.62	15.27	- 6.65
Oat hay period.								
Aug. 1-8.....	893	1.10	9.90	0.10	5.05	8.23	15.05	- 6.82
“ 8-15.....	945	1.15	10.86	0.08	4.93	8.23	15.87	- 7.64
“ 15-21.....	773	1.05	8.13	0.06	3.35	7.06	11.54	- 4.48

the alfalfa hay is considerably richer than oat straw in its content of the unknown factor influencing calcium assimilation and that the goat is less sensitive than the cow to its scarcity. In this connection it is well recognized that the goat is an animal of extraordinary persistent milking tendencies under most adverse conditions.

*Oat Straw Period.*—When feeding oat straw in place of alfalfa, casein was added to bring up the protein. As an example of a week's ration there were consumed during the second week 2,750 gm. of grain, 1,550 gm. of oat straw, and 186 gm. of casein.

During this period the calcium balance was always decidedly negative, but the decided deficit of the first week must be accepted with some reservations, as with a 6 to 8 fold decrease in calcium intake the lag of calcium excretion from the alfalfa period is decidedly contributory to the negative balance. As the pronounced negative balance persisted, there need, however, be no question as to the character of the change produced.

In the table, to bring out these facts, there are given in addition to other data both the weights of the feces and the percentage of calcium contained therein. These are important data and must be used in the interpretation of the results. A depression in the mass of feces with a rise in the percentage of calcium oxide with change of diet means nothing, but either a constancy in the fecal mass accompanied by a lowered percentage of calcium oxide or a depression in both means quite as much on a low calcium intake as does the actual establishment of a positive calcium balance. This latter will depend not only upon the kind of diet but also upon the amount of calcium in the ration.

*Green Oats Period.*—During this period, which ran for 4 weeks, fresh green oats (entire plant) were cut daily, sampled daily, and fed fresh, in amounts so that the calcium intake was approximately equal to that of the oat straw period; viz., 8 to 9 gm. On this uniform intake, though a positive balance was not established, probably because the intake was too low, the loss of calcium was reduced to approximately one-third of its former value. This has especial significance taking into consideration what was said in the preceding paragraph in regard to the importance of the relations of fecal mass to percentage of calcium contained therein, for while the fecal mass was reduced by the feeding of this succulent material, its percentage content of calcium was also reduced. Such a situation leaves no doubt as to the corrective effects brought about by the unknown factors of the fresh green roughage. Mere difference in solubility of the lime content was not the factor, as in harmony with our previous results where all the calcium in oat straw was found soluble in 0.05 N HCl, 95 per cent of

the calcium was extracted by digestion for 24 hours at 37° C. with a 0.2 per cent hydrochloric acid-pepsin solution.

*Oat Hay Period.*—During this period the green oats were substituted by the same material in the dried form as a hay, anticipating from what we expect occurs in farm practice, that this material would give about the same results as those secured with oat straw. For this purpose the oats were cut and dried in the diffused light of an attic lighted by skylights. Leaching by dew and rains was thus prevented but in addition changes induced by the fermentation of the curing process in the cock or mow were also eliminated so that the material was not strictly comparable to what is most commonly fed in general farm practice. Probably our expectations in regard to results obtained were also unwarranted, for feeding experiments with rats have shown that while oat straw contains little fat-soluble vitamine, this oat hay contained an abundance of it. So in certain relations at least, distinct differences in the effect of feeding these materials were to be expected.

3 weeks of record in the metabolism cage gave results unlike those obtained with oat straw but comparable to those secured with the green oats as seen in Table I. There was no rise in the fecal calcium elimination and no distinct difference in the calcium balance on practically the same intake. Possibly the factor influencing assimilation had not been greatly reduced in the oat hay as we dried it. Possibly our period of observation was too short. This phase will receive further study.

#### *Record of Animal 2.*

In the case of this animal our plan was to duplicate the procedure used with Animal 1. We did this in every respect but with the exception of omitting the dry alfalfa hay period. This animal was likewise milking and received the same materials as used in the first experiment. These materials consisted of the grain mixture and casein, with ordinary oat straw, green oats, or the attic dried oat hay. In all these experiments the amount of green oat hay allowed was made equivalent in dry matter to the dry matter of the oat straw. The records are shown in Table II.

These results duplicate in principle those secured with Animal 1. On the oat straw ration there was a high fecal calcium output and

a very pronounced negative calcium balance. On the green oats the dry weight of feces was practically the same as during the oat straw period but the percentage of calcium was reduced, making the total calcium elimination much less. Here a positive calcium balance was not established due to a low intake of calcium, but the degree of negative balance was greatly reduced. The effect with the oat hay was similar to our experience with Animal 1 and did

TABLE II.  
*Record of the Calcium Balance of Animal 2.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw period.								
	<i>gm.</i>	<i>percent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
June 17-24.....	1,547	1.50	23.26	0.03	10.86	13.34	34.15	-20.81
" 24-July 1....	1,578	1.44	22.84	0.03	8.48	13.57	31.35	-17.78
July 1-8.....	1,681	0.89	14.99	0.08	7.70	13.89	22.77	- 8.88
" 8-12.....	968	0.96	9.35	0.04	4.48	7.91	13.87	- 5.96
Green oats period.								
July 12-19.....	1,368	0.80	10.99	0.16	7.07	12.47	18.22	- 5.75
" 19-26.....	1,109	0.78	8.75	0.05	7.30	12.59	16.10	- 3.51
" 26-Aug. 2....	1,263	0.77	9.73	0.09	6.86	14.07	16.68	- 2.61
Aug. 2-8.....	1,037	0.75	7.80	0.04	5.58	11.47	13.42	- 1.95
Oat hay period.								
Aug. 8-15.....	1,509	0.59	8.90	0.05	5.75	13.83	14.70	- 0.87
" 15-21.....	1,261	0.65	8.24	0.04	4.69	11.86	12.97	- 1.11

not give the results expected; we expected an increased fecal calcium elimination and an increased negative calcium balance, but this did not result during the time of observations. Increased water intake was not a factor in these experiments since quite as much water was consumed on the oat hay ration as on the green oat ration making due allowance for its water content. For example, in a selected week on the oat hay ration the water consumption was 6,680 cc., on the green oats ration it was 5,940 cc.

*Record of Animal 3.*

Goat 3 was not a heavy producing animal, giving approximately only 200 gm. of milk per day when put on the basal ration of grain,

TABLE III.  
*Record of the Calcium Balance of Animal 3.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw period.								
	gm.	percent	gm.	gm.	gm.	gm.	gm.	
Oct. 11-18.....	1,284	1.44	18.59	3.31	0.39	16.01	22.29	-6.28
" 18-25.....	1,736	0.92	15.97	3.24	0.26	16.01	19.47	-3.46
" 25-Nov. 1....	1,647	0.99	16.35	3.28	0.25	16.01	19.88	-3.87
Butter fat period.								
Nov. 1-8.....	1,411	1.00	14.16	2.32	0.22	14.86	16.70	-1.84
" 8-15.....	1,283	1.09	14.05	1.81	0.17	9.42	16.03	-6.61
Orange juice period (60 cc. per day).								
Nov. 15-22.....	1,551	0.79	12.25	2.86	0.26	12.49	14.87	-2.38
" 22-29.....	1,291	0.75	9.74	1.74	0.29	14.32	11.77	+2.55
" 29-Dec. 6....	1,463	0.81	11.89	1.29	0.26	14.52	13.44	+1.06
Orange juice period (120 cc. per day).								
Dec. 6-13.....	1,408	0.85	11.98	2.05	0.29	14.71	14.32	+0.39
" 13-20.....	1,581	0.79	12.60	1.73	0.16	14.71	14.49	+0.22
Dried cabbage period (30 gm. per day).								
Dec. 20-27.....	1,467	0.81	11.92	1.63	0.24	14.64	13.79	+0.85
" 27-Jan. 3. ...	1,229	0.98	12.06	2.32	0.26	16.30	14.64	+1.66
Jan. 3-10.....	1,359	0.94	12.77	1.50	0.24	15.99	14.51	+1.48
" 10-17.....	1,371	1.01	13.94	1.65	0.22	15.99	15.81	+0.18
" 17-24.....	1,350	0.99	12.19	2.15	0.10	10.33	14.44	-4.11
" 24-31.....	1,119	1.11	12.43	1.39	0.12	14.56	13.94	-0.62
Feb. 1-7.....	1,221	1.18	14.42	1.44	0.19	15.75	16.05	-0.30
" 7-14.....	1,325	1.02	13.62	2.64	0.09	15.39	16.35	-0.96
Raw cabbage period (300 gm. per day).								
Feb. 14-21.....	1,383	0.94	13.12	2.22	0.07	14.71	15.41	-0.70
" 21-28.....	1,328	0.97	13.01	2.12	0.06	14.70	15.20	-0.50
" 28-Mar. 7. ..	1,357	0.99	13.52	3.26	0.05	15.39	16.84	-1.45
Mar. 7-14.....	1,447	0.93	13.48	2.53	0.05	14.34	16.07	-1.73

TABLE III—*Continued.*

Date	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Milk CaO.	Intake CaO.	Output CaO.	Balance.
Raw cabbage period (1,000 gm. per day).								
Mar. 14-21.....	1,019	1.12	11.47	2.19	0.08	14.01	13.74	+0.27
“ 21-28.....	829	1.47	12.23	3.36	0.08	13.65	15.67	-2.02
“ 28-Apr. 4....	928	1.30	12.12	2.77	0.07	14.87	14.96	-0.09
Apr. 4-11.....	870	1.51	13.21	2.96	0.05	13.73	16.22	-2.49
Cod liver oil period (5 cc. per day).								
Apr. 11-18.....	887	1.68	14.96	3.97	0.06	17.15	19.00	-1.85
“ 18-25.....	892	1.18	10.52	3.35	0.06	13.88	13.94	-0.06
Cod liver oil period (10 cc. per day).								
Apr. 25-May 2....	883	0.98	8.72	2.96	0.05	12.67	11.73	+0.94
May 2-9.....	670	0.95	6.39	3.38	0.05	9.83	9.52	-0.31
“ 9-16.....	Discontinued; not eating.							

casein, and oat straw. Her daily consumption was 600 gm. of grain, 40 gm. of casein, and 200 gm. of straw. On the ration, she rapidly went into negative calcium balance (see Table III).

Our results with green material, in these trials with green oats and in former work with mixed grasses, were so consistent that we believed that we were warranted at this stage of our experimentation to attempt to determine what factor in the green materials was operative in facilitating calcium retention. For this purpose our basal ration was supplemented successively with butter fat, orange juice, dried cabbage, fresh cabbage, and cod liver oil, all of which are materials which are well known to be rich in the fat-soluble vitamine or the antiscorbutic vitamine. These were selected not because we were inclined to the opinion that the factor we were dealing with was necessarily either one of these, but because we believed that this selection gave us a sufficient range of variables, including two well known factors with the possibility of the inclusion of others, so that the effect of the green oats might be duplicated. The relation of the water-soluble vitamine to this problem of calcium assimilation we felt free to disregard as our basal ration was, we believe, amply supplied with this factor.

*Butter Fat Period.*—In this period we gave 45 to 60 gm. daily of a clear filtered butter fat reducing the grain allowance by an amount equivalent in energy to the butter fat added. Butter fat feeding was continued 2 weeks. With this animal its ingestion affected the appetite adversely and milk production fell off very perceptibly, decreasing to approximately 50 cc. per day. In the short time of observation, the butter fat had no effect on the percentage of calcium eliminated by the feces, and the negative calcium balance was quite as large as on the basal ration. However, our data with butter fat are entirely too limited to give us an opinion as to whether or not it possessed any specific therapeutic value in influencing calcium assimilation.

*Orange Juice Period.*—Observations were next made with orange juice plus the basal ration. 60 cc. of orange juice were given daily for 3 weeks and then the dosage was increased to 120 cc. per day for 2 weeks. We had no difficulty in getting this animal to consume the orange juice with a complete recovery of appetite from the depressed condition experienced in the butter fat period. Milk flow was not completely restored although the daily volume now reached about 100 cc. as compared with but 50 cc. in the butter fat period. The calcium percentage in the feces was slightly but perceptibly decreased, and with the lowered milk flow the net result was a positive calcium balance. From these data alone one would be inclined to ascribe to the antiscorbutic vitamine some influence on calcium assimilation, but when, as will be shown later, no such positive calcium balances followed with the daily consumption of 1,000 gm. of fresh cabbage, also a very potent antiscorbutic material, and that with two other animals orange juice was not effective, there is left slight support for the assumption as made by Robb (10) that we were dealing with the antiscorbutic vitamine in green material as the factor influencing calcium assimilation. Possibly the orange juice we used at this time contained some of the food accessory which influences calcium assimilation.

*Dried Cabbage Period.*—Following the orange juice feeding came a long period where 30 gm. daily of dried cabbage (equivalent to about 300 gm. of fresh cabbage) were fed, plus the usual basal ration of grain mixture, casein, and oat straw. This dried cabbage was prepared by autoclaving at 15 pounds for 1½ hours and then



drying at 65-75° C. In this period there was a slow increase in the fecal calcium elimination, finally resulting in a negative calcium balance, although this did not take place until a lapse of 4 weeks of dried cabbage feeding. Since the behavior of this animal was indicating that the antiscorbutic vitamine might have some influence on calcium assimilation, in the next period of feeding we used fresh cabbage, feeding 300 gm. daily for 4 weeks and increasing this amount to 1,000 gm. daily for 4 more weeks with constant quantitative observations.

*Fresh Cabbage Period.*—During the period where we fed 300 gm. daily of raw fresh cabbage no reduction in the other constituents of the ration was made. The mass of feces remained practically the same as in the previous period and there was no appreciable change in the percentage of calcium in the feces. Negative calcium balance continued. When the fresh cabbage was increased to 1,000 gm. per day we reduced the straw intake from 170 to 25 gm. per day with the intention of keeping the fiber content of the ration approximately the same as in previous periods, and thereby holding the fecal mass constant. We did not succeed in this and consequently added ordinary filter paper, 60 gm. per day, for the purpose of increasing the fecal residue. With this goat the added paper did not raise the fecal residue.

The result of a lowered fecal residue during the 1,000 gm. of raw cabbage feeding was a marked increase in the percentage of calcium in the feces with the net result that just as much calcium continued to be excreted in the feces as in the previous feeding period, and a negative calcium balance continued. These results left no question as to the negative relation of the antiscorbutic vitamine to this phenomenon of calcium assimilation. While fresh cabbage or dried cabbage contains some fat-soluble vitamine (11), yet it is not particularly rich in this food factor; should it be established later that this vitamine is the one related to calcium assimilation it would be necessary to assume that cabbage was not sufficiently rich in this factor to bring about such an influence.

The failure to induce a positive calcium balance with such large amounts of cabbage is interesting when it is compared with the one positive effect of the orange juice administration. Both are rich in the antiscorbutic vitamine, and weight for weight they are

probably of approximately equal value as sources of the fat-soluble vitamine as shown by our feeding experiment with rats. Yet before attempting to make extensive analytical use of these discrepancies it is well to bear in mind that negative results must not be given too absolute a valuation as the recuperative elasticity of an animal in trials of this nature has decided limitations.

*Cod Liver Oil Period.*—The general recognition of cod liver oil as a successful therapeutic agent in rickets led us to use it in these experiments. It was emulsified with acacia gum and water and for 2 weeks this emulsion was administered in amounts equivalent to 5 cc. daily of the original oil. In the 3rd and 4th week of this period an equivalent of 10 cc. of oil was given daily. There was no influence on calcium assimilation in the 1st week, but in the 2nd week the fecal calcium oxide dropped from 1.68 to 1.18 per cent and in the 3rd week to 0.98 per cent with the output of feces practically the same as in the cabbage period. This influence of the oil resulted in establishing a positive calcium balance in the 3rd week, with a decrease in the calcium oxide of the feces from 14 to 8.7 gm. There was soon developed a distinct dislike for the oil and in the 4th week there occurred a loss of appetite with the result that the food intake was reduced in this week and a slight negative calcium balance resulted. It is safe, however, to conclude that cod liver oil was an effective agent in assisting calcium assimilation in this species.

#### *Record of Animal 4.*

*Basal Ration Period.*—While a milking goat receiving our ration of grain mixture, casein, and dry oat straw, responded readily with a negative calcium balance, we were not sure that such a reaction would follow with a dry animal. To determine this, Animal 4 was started on our basal ration October 11, 1920. She remained in positive calcium balance or equilibrium until the 2nd week in December, when a distinct negative calcium balance was assured. Feeding of this ration was continued until January 31, 1921, at which time the animal's appetite was becoming poor and her general condition apparently somewhat impaired. Her feces were hard and dry. She showed a negative calcium balance of 3.04 gm. of calcium oxide for the week. See Table IV for the record of results.

*Record of the Calcium Balance of Animal 4.*

[illegible]

*Orange Juice Period.*—On January 31, 1921, administration of 120 cc. of orange juice daily was begun. This resulted in some stimulation to appetite and increased food consumption, but there was no change in the amount of calcium assimilated and a negative calcium balance continued. On February 14, 1921, the amount of orange juice allowed was increased to 240 cc. per day, but even this amount did not decrease the fecal calcium elimination and the animal remained at negative calcium balance.

*Cod Liver Oil Period.*—On April 4, 1921, 5 cc. of cod liver oil per day as an emulsion with acacia gum and water were given. For the first 2 weeks of oil administration there was little influence on the percentage of calcium in the feces, but with decreasing appetite and decreasing fecal output the net result was a lowered calcium elimination. This situation continued into the 3rd week with a decreasing percentage of calcium in the feces, and an actual change to a positive calcium balance in this last period. After 3 weeks on a daily allowance of 5 cc. of cod liver oil, we increased the amount to 10 cc. but the appetite of the animal had become so poor and the intake of food so low that the results of the last 2 weeks mean little. The balance experiments for this animal do show, however, that a negative calcium balance can be established with a dry goat receiving our basal ration; that orange juice had no influence on calcium assimilation; and that cod liver oil in as low amounts as 5 cc. per day did effect a better retention of the calcium of the feed.

#### *Record of Animal 5.*

*Basal Ration Period.*—The record of Animal 5 was substantially a duplication of that of No. 4, both being non-producers. The results are recorded in Table V. After prolonged feeding (2 to 3 months) a negative calcium balance was established on our basal ration. This animal as well as No. 4 was not in the metabolism cage continuously as seen in the protocols, but received the basal ration through the entire period of observation whether in the cage or not.

*Orange Juice Period.*—On February 7, 1921, after we were positive that the animal was in negative calcium balance, 120 cc. of orange juice were given daily. This was continued for 6 weeks but without any appreciable change in calcium assimilation.

The mass of feces and percentage of calcium therein remained practically like that of the preceding period.

TABLE V.  
*Record of the Calcium Balance of Animal 5.*

Date.	Dried feces.	CaO in feces.	Feces CaO.	Urine CaO.	Intake CaO.	Output CaO.	Balance.
Oat straw ration.							
	gm.	per cent	gm.	gm.	gm.	gm.	
Oct. 11-18.....	1,190	0.87	10.37	0.12	11.30	10.49	+0.81
" 18-25.....	1,068	1.15	12.28	0.38	11.30	12.66	-1.36
" 25-Nov. 1....	1,074	1.56	16.70	0.74	11.30	17.44	-6.14
Nov. 1-8.....	1,056	0.85	9.01	0.59	11.30	9.60	+1.70
" 8-15.....	1,121	0.74	8.32	0.59	11.30	8.91	+2.39
Dec. 6-13.....	1,029	1.09	11.28	0.06	11.22	11.34	-0.12
" 13-20.....	1,316	0.96	12.72	0.07	11.22	12.79	-1.57
Jan. 17-24.....	1,297	0.92	12.23	0.08	10.92	12.31	-1.39
" 24-31.....	1,086	1.00	10.91	0.08	10.92	10.99	-0.07
" 31-Feb. 7....	1,082	1.05	11.44	0.07	10.89	11.51	-0.62

Orange juice period (120 cc. per day).

	gm.	per cent	gm.	gm.	gm.	gm.	
Feb. 7-14.....	1,229	0.97	11.92	0.07	10.28	11.99	-1.71
" 14-21.....	1,159	0.91	10.64	0.07	11.06	10.71	+0.35
" 21-28.....	1,071	0.92	9.93	0.05	11.06	9.98	+1.08
" 28-Mar. 7....	1,140	0.95	10.90	0.03	11.06	10.93	+0.13
Mar. 7-14.....	1,241	0.94	11.69	0.04	11.06	11.73	-0.67
" 14-21.....	1,128	1.01	11.39	0.03	11.06	11.42	-0.36

Cod liver oil (20 cc. per day) for 2 days. Off feed.

	gm.	per cent	gm.	gm.	gm.	gm.	
Apr. 4-11.....	1,007	0.90	9.11	0.07	9.64	9.18	+0.46

Cod liver oil period (5 cc. per day).

	gm.	per cent	gm.	gm.	gm.	gm.	
Apr. 11-18.....	894	0.84	7.52	0.06	9.64	7.58	+2.06
" 18-25.....	846	0.75	6.42	0.06	8.65	6.48	+2.17
" 25-May 2....	804	0.65	5.22	0.04	5.99	5.26	+0.73

*Cod Liver Oil Period.*—On March 21, 1921, we began the administration of 20 cc. of cod liver oil per day. This was continued for 2 days and so completely upset the appetite of the animal that further administration of it was discontinued. At this

point, we again reverted to the basal ration, only beginning quantitative collection of the excreta from the basal ration on April 4 to 11. This week showed a positive calcium balance which may be interpreted as the residual effect of the cod liver oil given earlier, but the decrease in fecal calcium was not marked. After reestablishing the animal on the basal ration, we continued to give 5 cc. of cod liver oil daily as an emulsion. The effect of this in reference to calcium assimilation was gradual but positive. The fecal residue decreased somewhat, while the percentage of calcium in the feces was decreased from 0.90 to 0.65 per cent, giving a distinct positive calcium balance. However, the long cod liver oil administration gradually impaired the food intake of this animal and the experiment was discontinued. The records of this animal are in accord with those of No. 4. With the basal ration a negative calcium balance was established; the added orange juice did not consistently influence calcium assimilation; the cod liver oil did decrease the calcium assimilation in the feces with the production of a distinct and continued positive calcium balance.

#### SUMMARY.

1. Experiments with goats, milking and dry, show that there is something in fresh green oats as compared with a dry oat straw which increases the amount of calcium assimilated. The oat hay, dried out of direct sunlight, but in a fairly well lighted attic, seemed to retain the properties of the fresh green oats that we were studying.

2. Orange juice administered in generous quantities (120 to 240 cc. per day) had no consistent effect on calcium assimilation.

3. Raw cabbage (1,000 gm. per day) or dried cabbage, had no influence on calcium assimilation. These data eliminate the antiscorbutic vitamine as a factor in calcium assimilation and conform with clinical experience in rickets.

4. Cod liver oil (5 to 10 cc. per day) consistently changed negative calcium balances to positive balances.

5. Our limited data show that the same factor affecting calcium assimilation and resident in green oats and grasses is present in cod liver oil.

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# A METHOD FOR THE DETERMINATION OF SUGAR IN NORMAL URINE.

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In a previous paper<sup>1</sup> we described a procedure for the determination of sugar in normal urines based upon preliminary removal of the nitrogenous urinary constituents by means of mercury nitrate in the presence of sodium bicarbonate. The sugar was then determined in the filtrate by the use of picric acid and alkali under definite conditions. This method has been of considerable service. Frequent checkings by comparative determinations made upon the same filtrates by the Allihn gravimetric method which we have carried out have convinced us that the method is accurate for both unfermented and fermented urines. Duplicates by the colorimetric and the Allihn method practically always agree with 0.02 per cent on the sugar content of the urines.

The mercuric nitrate method has, however, a serious drawback in the laborious technique involved, which has interfered with the general usefulness of the method. We have, therefore, constantly kept in mind the development of a procedure which should permit of the determination of small quantities of sugar in urine without the use of so troublesome a technique. The method described in the present paper meets this requirement. It has been in constant use in our laboratories and elsewhere for about 2 years, and we now feel quite sure of its accuracy and reliability under widely varying conditions. While the present method retains the use of picric acid, it seems probable, as will be pointed out later, that the actual reaction takes place between the sugar and an unidentified derivative of picric acid.

<sup>1</sup> Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.



The chief interfering substances in urine as regards the determination of sugar by picric acid (or indeed by other methods as well) are creatinine and creatine. In constructing the present method we have, therefore, had in mind primarily the elimination of interference by these compounds. The procedure developed has accomplished this end. It appears that interference by minute traces of other substances has been eliminated as well.

It has been noted in the literature that acetone causes a partial fading of the color resulting from the interaction of creatinine and picric acid in the presence of sodium hydroxide. Preliminary experiments convinced us that if the reaction between picric acid and sugar could be made to go quantitatively in the presence of hydroxide instead of carbonate, which we have had to employ heretofore, it would be possible to utilize the acetone effect on the creatinine-picric acid product, so that sugar could be determined in the presence of large amounts of creatinine or creatine. We have found that if a low concentration of picric acid is used the reaction with sugar proceeds quantitatively in the presence of sodium hydroxide. It has further been found that by addition of suitable quantities of acetone to such a mixture it is possible to destroy completely the color due to creatinine or creatine, without a serious effect on the color developed through the action of the sugar.

Before describing the exact technique of our method the following points may be mentioned as of some general interest.

The impression which quite commonly prevails that picramic acid is the colored product resulting from the interaction of creatinine and picrate in alkaline solution is incorrect. We have isolated many grams of the product of the action of picrate and creatinine, and in physical properties and stability it is quite different from picramic acid. The product of the creatinine reaction is a bright, carmine powder, which is unstable even in the dry form when exposed at all to light. When so exposed it rapidly becomes lemon yellow in color, as though reoxidized to picric acid. Analyses of the product indicate that its empirical formula is quite close to, or identical with, that of picramic acid. Nevertheless, the products are, as noted, entirely different. The procedure proposed in the present paper serves also to demonstrate the difference between the reaction products of sugar and

of creatinine with picric acid. In our process we add picric acid, alkali, and acetone to the solution, and heat. A color develops due to the acetone alone, which color fades rapidly so that in a minute or two the mixture again assumes the color of picric acid. If creatinine or creatine be present the color developed during the early stage of the heating is much intensified, so that in such a case the solution may become very intensely colored during the first minute or two of heating. Within about a minute and half, however, the color begins to fade just as does the color due to acetone alone, so that after 5 minutes of heating such solutions can scarcely be differentiated from a blank. Upon subsequent dilution these solutions have only a light yellow color as found in a blank. If sugar is present the color due to this develops more slowly than the color due to acetone or to creatinine, and does not fade or change with continued heating for at least 45 minutes. It is interesting to note that the reaction is apparently not between the sugar and the picric acid. This is indicated by the fact that during the first part of the heating in the presence of acetone, and before the sugar apparently begins to react with picric acid, it can be shown that this latter substance has been completely destroyed by the acetone. If sugar (or creatinine) be added after the heating with acetone has been carried on for a short time no color whatever develops, showing that the solution no longer contains any picric acid. If the solution after heating with the acetone until the color has faded to light yellow be acidified with hydrochloric acid and warmed, the mixture turns deep red-brown in color, and a dark precipitate forms. We have been unable to identify this product. It would seem probable that in our method the sugar reacts with an intermediate reaction product between acetone and picric acid. Creatinine will not react with this product, but reacts quickly with the original picric acid to form a compound which is not stable in the presence of acetone. It is interesting that by this adjustment of conditions it is possible to determine accurately sugar in the presence of three or more times its weight of creatinine, when under ordinary conditions creatinine yields with picric acid about three to five times as much color as does an equal weight of glucose. Indeed the reaction now proposed appears to be perhaps the most specific reduction test available for

sugar. Certainly when applied directly to urine the reaction gives more accurate results for sugar than does any other test. One may have three or four times as much creatinine or creatine present as of sugar without affecting the results. Larger amounts of creatinine may cause a slight lowering of the figure for the sugar. Hydrogen sulfide, which readily reduces picric acid in alkaline solution under ordinary conditions may be present in relatively very large amounts (1 cc. or more of a saturated solution) without affecting the results. We had hoped that the reaction could be applied directly to urine with satisfactory results. The figures obtained in this way are, however, slightly too high, as will be evident from an inspection of Table II. We have therefore adopted a procedure of preliminary shaking of the urine with purified bone-black, which we have found removes the trace of unknown interfering substance. The bone-black used is prepared as follows. 250 gm. of commercial bone-black<sup>2</sup> are treated with about 1.5 liters of dilute hydrochloric acid (1 volume of concentrated acid diluted with 4 volumes of water) and the mixture is boiled for about 30 minutes. The bone-black is now filtered off on a large Buchner funnel and washed with water (preferably hot) until the washings are neutral to litmus. The product is then dried and powdered. The highly absorbent animal charcoals on the market should be avoided in this connection. Commercial bone-black should be used, and the final product should be tested by shaking a portion (15 cc.) of a glucose solution containing 1 mg. of the sugar in 2 cc. of water with 1 gm. of the bone-black and determining the sugar in the filtrate. There should be no detectable absorption of the sugar.

Following is the procedure for the determination of sugar in urine. The urine should be diluted so that the specific gravity does not exceed 10.25 to 10.30. 15 cc. of the urine are treated with about 1 gm. of bone-black (smaller quantities of both may be used if desired) and the mixture is shaken vigorously occasionally for a period of 5 to 10 minutes. The mixture is then filtered through a small dry filter into a dry flask or beaker. The volume

<sup>2</sup> We have employed commercial bone-black supplied by Eimer and Amend. Different samples supplied over a period of 2 years have all yielded satisfactory results. The crude bone-black must not be used without purification.

of this filtrate to be used in the determination will depend upon its sugar content, but should never exceed 3 cc. Such a volume should be used as will contain about 1 mg. of sugar. Usually 1 to 2 cc. is the right amount. The proper volume of the urine filtrate is measured into a large test-tube which is graduated at 25 cc., and if the volume used was less than 3 cc. enough water is added to make the volume exactly 3 cc. Now add exactly 1 cc. of 0.6 per cent picric acid solution (best prepared from dry picric acid) and 0.5 cc. of 5 per cent sodium hydroxide solution. Just before the tube is ready to be placed in boiling water add 5 drops of 50 per cent acetone (this should be prepared fresh every day or two by diluting some pure acetone with an equal volume of water) taking care that the drops fall into the solution and not on the sides of the tube. Shake the tube gently to mix the contents, and place immediately in boiling water and leave for 12 to 15 minutes. The standard solution should be simultaneously prepared by treating 3 cc. of pure glucose solution (containing 1 mg. of the sugar) exactly as described for the unknown solution and heating simultaneously. The pure glucose solution containing 1 mg. of the sugar in 3 cc. of solution will keep indefinitely if preserved with a little toluene. We have not been able to find a colored solution which matches the colored product of the reaction and which is permanent.

In connection with the use of the method attention may be called to the following points. The quantity of the picric acid solution used must be measured with exactness, just as are the unknown and standard sugar solutions. Slight variations in the alkali are not so important. Adding the same number of drops (about 10) to each of the tubes from the same pipette is sufficient. The acetone solution should be added last, and the tubes placed in the water bath within about a minute afterwards. The diluted acetone undergoes some peculiar change on standing which makes old solutions yield somewhat irregular results. It is therefore best to prepare the acetone solution fresh every day or two.

Each solution should be so added that it falls into the bottom of the tube, and does not hit the sides. Standard and unknown must correspond in sugar content within reasonable limits. For a 1 mg. standard satisfactory results can be obtained for an unknown solution containing between 0.75 and 1.75 mg. of sugar.

With wider variations between unknown and standard results are not so good, particularly when the quantity of sugar is low. If less than 0.7 mg. of sugar is present in the unknown it is better to have a standard solution containing 0.5 mg. of sugar in 3 cc., and to dilute both unknown and standard to 12.5 instead of to 25 cc.

TABLE I.

*Comparative Results for Sugar in Normal Urine by the Mercuric Nitrate-Picric Acid Method and by the New Procedure.*

Sample No.	Mercuric nitrate-picric acid method.		New method.	
	Before fermentation.	After fermentation.	Before fermentation.	After fermentation.
Dog urine.				
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.073	0.022	0.062	0.024
2	0.095	0.055	0.087	0.042
3	0.065	0.041	0.063	0.042
4	0.056	0.035	0.042	0.017
5	0.111	0.057	0.092	0.059
6	0.107	0.034	0.094	0.034
7	0.079	0.036	0.081	0.037
8	0.084	0.034	0.079	0.027
Human urine.				
1	0.147	0.083	0.140	0.075
2	0.077	0.033	0.069	0.033
3	0.111	0.065	0.091	0.056
4	0.086	0.043	0.077	0.043
5	0.065	0.048	0.068	0.046
6	0.064	0.034	0.068	0.046
7	0.109	0.050	0.119	0.062
8	0.211	0.068	0.220	0.060

Where the method is intelligently carried out it is very simple, and yields results of a high degree of accuracy. We have studied the procedure in detail as regards recovery of added sugar, and the determination of sugar in urine with and without creatinine and creatine addition. The results have been wholly satisfactory.

About fifty determinations have been made comparing results obtained by the new method with those given by the mercuric

nitrate procedure. A few of these results are recorded in Table I. It will be noted that the new procedure gives consistently slightly lower figures than does the old, but upon the whole the agreement is excellent between the two methods. The new method applied to the mercuric nitrate filtrates gives no lower figures than when the bone-black is employed, showing that there is no nitrogenous constituent of the urine which interferes with the method.

TABLE II.

*Comparison of the Figures Obtained for the Sugar Content of Urine with and without Preliminary Treatment with Bone-Black.*

Sample No.	Sugar.	
	With bone-black.	Without bone-black.
Dog urine.		
	<i>per cent</i>	<i>per cent</i>
1	0.078	0.100
2	0.074	0.096
3	0.072	0.096
4	0.096	0.133
5	0.110	0.144
6	0.060	0.077
7	0.119	0.168
Human urine.		
1	0.083	0.110
2	0.066	0.110
3	0.181	0.195
4	0.037	0.053

For clinical purposes the use of bone-black might be omitted if desired. Under such conditions figures will be obtained which are about 0.03 to 0.04 per cent too high. Table II shows some comparative figures with and without the use of bone-black.

We are studying the question of the adaptation of the new procedure to the determination of sugar in tissues and in blood. On account of its high degree of specificity for sugar the new procedure may prove to be of advantage in these determinations.



# CHEMICAL DEVELOPMENT OF THE OVARIES OF THE KING SALMON DURING THE SPAWNING MIGRATION.

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(Received for publication, May 24, 1921.)

Analytical data are presented here of the chemical development of the ovaries of the king salmon during the prolonged stage of its migration. Active development of the sex gonads takes place chiefly after the entrance of the salmon into fresh water. The gonad growth, therefore, occurs in the absence of an intake of food; *i.e.*, at the expense of stored materials on hand at the beginning of the migration. The migration and the time on the spawning beds preliminary to the act of spawning takes 2 to 4 months or even more. The ovaries increase in weight, during the migration of the spring run on the Columbia River, from 200 to 300 gm. at the beginning to as much as 2,500 gm. at the end.

Coincident with the growth of the ovary there is the expenditure of much dynamic energy in the migration. This dynamic energy is derived primarily from the potential energy of the excess of proteins and fats stored in the muscles in large amounts and to some extent in other organs and tissues. There is an absolute loss of mass of the muscle tissue during the migration amounting to some 45 per cent of the total. The muscle tissue left is much poorer in both proteins and fats. The fats alone drop from 20 per cent per unit mass at the beginning of the journey to less than 2 per cent at the spawning. The percentage of protein calculated on a protoplasmic basis, decreases from 20 per cent at the close of feeding to 14 per cent at the spawning. In other words, of 100 per cent of protein of muscle per unit mass of protoplasm at the beginning of migration, 30 per cent has disappeared at the spawning. Computing the loss of protein and of fat in



the tissue remaining at the spawning time and deducting that from the 45 per cent total loss during the migration, it appears that some 25 per cent or more of the muscular tissue as such has totally disappeared. These losses provide a source for a large amount of potential energy as well as for the materials that reappear in the ovaries. The foundation for these facts has been set forth in a previous publication.<sup>1</sup> They bear directly on the problem of food storage in the developing ovaries, a process that takes place coincident with the retrogressive changes in the muscle and other tissues of the salmon.

The problems undertaken in this paper are: first, the mass change in the total growth of the ovary; second, the percentage composition of the ovarian protoplasm; third, the proportionate amounts of typical stored food materials found in the eggs of the salmonoid fishes at different stages of the spawning journey; and finally, the history of the phospholipins present in such rich quantities in all eggs.

### *Historical.*

Few references have been found in the literature giving the complete analysis of the constituents of fish eggs and ovaries, and none of the ovaries of American fishes except that of Atwood's analyses of food fishes in which he gives the composition of shad roe.<sup>2</sup> Earlier preliminary reports of the work detailed in this paper represent the only data available on the chemical development of the ovaries of American fishes.<sup>3</sup> In Europe, Miescher's classic studies of the Rhine salmon<sup>4</sup> and the studies of Paton of the Scottish salmon<sup>5</sup> give data of the composition of the salmon roe as regards the store of simple and compound fats which they regard as the chief food source in the yolk.

Buttenberg<sup>6</sup> investigated the chemistry of different caviars. He also gives two analyses of fresh sturgeon roe. The averages for fresh sturgeon roe are: water 62.3, ash 1.71, nitrogenous substances (proteins) 22.8, and fat 9.9 per cent. The ash is higher than in salmon roe, the proteins are lower, and the fat content is comparable to that of the eggs of fasting salmon obtained at the spawning beds.

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<sup>1</sup>Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

<sup>2</sup>Atwater, W. O., *Rep. U. S. Com. Fish and Fisheries*, 1888, 679.

<sup>3</sup>Greene, C. W., *J. Biol. Chem.*, 1918, xxxiii, p. xiii.

<sup>4</sup>Miescher, F., *Schweizerischer Fischerei-Ausstellung in Berlin*, 1880.

<sup>5</sup>Paton, D. N., *Rep. Fishery Bd. Scotland*, 1898, iv, 63.

<sup>6</sup>Buttenberg, P., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1904, vii, 233.

Rimini<sup>7</sup> also analyzed preserved fish roe, including different types of sturgeon caviar. The sodium chloride ran from 1.2 to 11 per cent, correspondingly raising the mineral content and lowering the percentages of the organic fractions. The fats of caviar ran from 14 to 28 per cent, the latter in a sample containing only 16.5 per cent of water. There is in fact no common basis of comparison between the composition of caviar as against fresh roe.

Tangl and Farkas<sup>8</sup> studied the chemical changes in the developing trout eggs. They make the following comparisons:

	Undeveloped eggs.		Developing eggs.
Water .....	66.67	66.08	65.6
Solids .....	33.33	33.92	34.94
Fat.....	0.73	7.58	7.98 Saponified fats.

These analyses indicate that the eggs lost weight, water, and energy (calories), but gained fat during incubation. The author compares the loss in trout, chicken, and spider eggs, showing the proportionate loss in each during the incubation. The trout loses the least in total weight, 5.6 per cent; the chicken egg, 17 per cent; and the spider egg, 26 per cent.

Solberg<sup>9</sup> analyzed "Dorsch" roe (Norway), finding water 66.03 per cent, proteins 29.92 per cent, amino-acids 4.67 per cent, fat 2.26 per cent, and ash 2.16 per cent.

Kojo<sup>10</sup> gives the following analyses of the white and the yolk of the chicken's egg.

	White.	Yolk.
Water .....	87.71	49.73
Solids .....	12.29	50.27
Ash .....	0.4	1.44
Glucose .....	0.55	0.27
Nitrogen .....	1.75	2.49

The comparison between the salmon egg and the hen's egg is in the yolk. The percentage of water and total solids checks closely but the yolk of the hen's egg contains only a trifle more than half as much protein, three times as much lecithin, and somewhat more neutral fat than is contained in the salmon egg. The

<sup>7</sup> Rimini, E., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1904, vii, 232.

<sup>8</sup> Tangl, F., and Farkas, K., *Arch. ges. Physiol.*, 1904, civ, 624.

<sup>9</sup> Solberg, E., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1908, xvi, 364.

<sup>10</sup> Kojo, K., *Z. physiol. Chem.*, 1911, lxxv, 1.

important point made in Kojo's analyses is the demonstration of glucose; namely, 0.27 per cent in the yolk and 0.55 per cent in the white. Glucose is also constantly present in the salmon egg, but only to the extent of 0.09 per cent.<sup>11</sup>

### *Collecting Stations.*

The king salmon readily available for a study of this nature are the schools which migrate up the Columbia River to spawning beds in the cold waters of the Cascades and the Rocky Mountain rivers, and those which make similar migrations in the Sacramento River in California to spawning beds in the head waters of streams arising on the slopes of Mt. Shasta. Both series have been studied but the present report is based on a collection of salmon from the Columbia River basin made in the summer of 1908.

In this series, seventeen salmon chosen from five different stations of the Columbia River and its tributaries have been analyzed in detail. The stations chosen are in order, Ilwaco at the mouth of the Columbia River; Warrendale in the Cascades, 135 miles up the Columbia; Seufert's fishery on The Dalles of the Columbia, 210 miles up; and at Ontario on the Snake River, 700 miles above the mouth of the Columbia. The spawning fish were obtained from Cazadero, some 30 miles above Portland on the San Lorenzo River. The spawners were undoubtedly of the spring migration which entered the San Lorenzo River through the Willamette River. The other samples are from spring and summer migrants.

### *Methods of Sampling and of Analysis.*

The method of selecting salmon types and of taking and preserving samples followed in this series of fish has already been described. The analytical procedures are the same as used on muscles and are presented in a previous paper.<sup>1</sup> Samples of ovaries and of free eggs are rather difficult to pulverize and require greater precaution in extractions, especially of the lecithins. The method in brief was as follows. The sample preserved in alcohol was transferred to a Gooch crucible and extracted in a Soxhlet modified to insure extraction at the boiling point of the

<sup>11</sup> Greene, C. W., in press.

solvent.<sup>12</sup> The sample was then extracted first in alcohol, then ether, then alcohol, and finally in ether, 6 to 8 hours each. The residue was finely pulverized after the first ether extraction. The insoluble residue was dried to constant weight at 105°C. and given six extractions in distilled water. The water-soluble was evaporated and dried to constant weight, then ashed and weighed. The alcohol-ether-water-insoluble was obtained by difference and the organic extractives also by difference. The alcohol and ether were driven off the alcohol-ether-soluble fraction and the lecithins thrown down with acid chloroform-water by the method of Koch. The emulsion of lecithins and fats was oxidized, and the phosphorus determined by the official gravimetric method. The phospholipins were computed by the lecithin-phosphorus factor. Several analyses of phospholipins of critical samples were lost during the oxidations and the determinations do not reach our ideal of accuracy for the purposes. The organic extractives in the alcohol-ether-soluble fraction and from the alcohol-ether-insoluble fractions and the corresponding ashes were determined separately, but are combined in Table I. The waters were determined on a separate sample, and the neutral fats computed by difference.

#### *Growth of the Ovary.*

The growth of the ovaries in the salmon takes place chiefly during the migration. This fact is shown from the weights of the ovaries of fishes from the different stations in this series and in data obtained in the Sacramento River basin of California. The contrasts would be much more striking if sea-run fish could have been secured for the Columbia River series comparable to feeding salmon from Monterey Bay or Bolinas Bay, California.

The weights of the ovaries of fish taken in July and August from the lower Columbia River fishing stations vary from 501 to 747 gm. which compare with similar weights at tide-water on the Sacramento. The weights from the spawning beds are from 775 to 2,243 gm. for ovaries, 2,596 for ripe ova and ovaries. This represents a maximum increase of 400 to 500 per cent and is an enormous storage of food substances. Fish of the size indicative of approaching maturity, 8 to 10 kilos body weight,

<sup>12</sup> Greene, C. W., *J. Biol. Chem.*, 1909-10, vii, 503.

TABLE 1.

Showing the Length, Weight, and Date of Collection, the Ovarian Weight, and the Groups of Chemical Constituents of Salmon Ovaries Collected from Five Stations, Representing Stages of the Spawning Migration, Columbia River, Season of 1908.

[illegible]

Ontario, Ore.	Sept.	61, 312	940	7, 490	696	1, 496	53.35	62.04	65.37	96.26	29.56	36.30	57	3.9	8.5	4.6	14.0	30	01	45	3.101	68.0	931.99	1.08	
	"	71, 315	1, 076	10, 410	916	1, 498	53.35	62.67	66.37	33.26	13.56	02.30	70	3	4	7.4	4.0	14.9	31	91	27	731.50	0.92	1.97	1.07
	"	91, 321	1, 076	14, 900	1, 899	1, 523	54.59	61.41	45.41	38.59	28.57	62.92	32.14	3.5	7.8	4.0	11.1	24.5	1	52	3.341	710.64	1.41	0.72	
	"	111, 326	1, 034	12, 840	1, 282	1, 524	54.59	60.84	45.41	39.16	28.50	62.72	31.77	4.4	9.8	4.9	10.3	22.6	1	52	3.351	700.66	1.45	0.73	
	"	Aug. 25	1, 294	957	9, 000	1, 775	1, 553	54.86	64.66	45.14	35.34	26.58	58.89	31.33	1.7	3.8	2.0	15.1	33.6	1	12	2.501	330.53	1.17	0.62
Cazadero, Ore.	"	261, 296	1, 054	12, 910	1, 860	1, 555	54.86	64.39	45.14	35.61	26.52	58.75	31.13	1.7	3.7	1.9	14.5	32.0	1	42	3.131	660.71	1.56	0.83	
	"	261, 297	966	10, 515	2, 243	1, 580	54.64	63.57	45.36	36.43	27.02	59.58	31.40	2.5	5.5	2.9	14.0	31.0	1	41	3.111	640.64	1.42	0.75	
	"	271, 299	887	8, 145	1, 679	1, 429	57.24	65.23	42.76	34.77	25.89	60.55	30.00	2.3	3.1	2.2	12.2	28.6	1	57	3.671	750.76	1.77	0.86	
	"	"	"	"	"	1, 452	57.68	65.60	42.32	34.40	26.66	63.01	30.33	1.5	3.5	1.7	12.1	28.5	1	41	3.341	600.66	1.56	0.75	
	"	"	"	"	"	1, 453	57.68	65.04	42.32	34.96	26.45	62.50	29.83	2.4	5.6	2.6	11.3	26.7	1	44	3.421	630.71	1.67	0.80	
	"	"	"	"	"	1, 460	59.96	67.00	40.04	33.00	24.85	62.07	27.77	2.6	6.6	2.9	10.5	26.2	1	13	2.831	270.90	2.25	1.01	
	"	"	"	"	"	1, 462	59.96	66.01	40.04	33.39	24.87	62.11	27.63	3.6	8.6	3.9	9.1	21.7	1	37	3.271	510.65	1.55	0.77	
	"	"	"	"	"	1, 473	58.19	64.02	41.91	35.98	27.06	64.73	29.78	3.6	8.6	3.9	9.1	21.7	1	37	3.271	510.65	1.55	0.77	
						1, 474	58.19	64.08	41.91	35.92	26.95	64.46	29.68	3.6	8.6	3.9	9.1	21.7	1	37	3.271	510.65	1.55	0.77	
						1, 474	58.19	64.08	41.91	35.92	26.95	64.46	29.68	3.6	8.6	3.9	9.1	21.7	1	37	3.271	510.65	1.55	0.77	

\*The total weight was 2,560 gm., the ripe ova free in the body cavity 1,860 gm., and the ovary with adherent ova 736 gm.

†Free ova = 1,679 gm. Ovary = 71 gm. Egg fluid = 179 gm. Total = 1,929 gm.

feeding at Monterey Bay in July, have ovaries of from 132 to 150 gm. The smallest ovaries collected at the mouth of the Columbia and at tide-water on the Sacramento River have reached 500 gm. and more in weight.

The development of the ovaries of salmon at the time of beginning the migration varies with the season. The August salmon of the mouth of the Columbia at Ilwaco are more mature as regards the sex gonads than are those from the same station that begin the migration in the spring. It is the spring run that spawn in the San Lorenzo River and apparently that migrate up the Snake River. The August fish spawn in the White Salmon River and other streams of the Cascade Mountains.

The observations seem to justify the conclusion that from 90 to 95 per cent of the total weight of the mature ovaries of the king salmon is acquired during the spawning migration; *i.e.*, while the salmon is in fresh water and not taking food. It is a unique case of synthesis and growth of the tissues of the gonads while all other organs are decreasing by a process analogous to tissue starvation.

### *The Chemistry of the Salmon Ovaries.*

The analytical data presented in this series follows the quantitative distribution of the proteins, lipins, phospholipins, extractives, inorganic salts, and water in the ovaries and in ripe ova. The distribution of the nitrogen and of the various phosphorus fractions has been followed, but the data is not now presented.

The data have been calculated on the three bases used in presenting results of the analyses of muscle. These are: first, per cent of the wet sample as collected; second, per cent of the dry or water-free sample; and third, per cent of the fat-free sample. The stored food material in the egg is out of all proportion to the active protoplasm of the germ cell or cells. This is obvious when one remembers that the protoplasm of the ovum itself is microscopic whereas the total egg is 6 to 7 mm. in diameter. Neither of the above classifications therefore can be considered as representative of the composition of the ovarian protoplasm. The data represent only the composition of stored food material, however computed.

*The Mature Ova.*

Two of the four female fish sampled at the spawning grounds contained mature ova separated from the ovarian tissue and floating free in the ovarian fluid in the body cavity. The composition of these two samples of mature eggs is as follows in terms of per cent of the total sample: water 57.68 and 58.19 per cent; total solids 42.32 and 41.91 per cent; protein 26.56 and 27.01 per cent; phospholipins 1.9 (probably low) and 3.6 per cent; neutral fats 11.70 and 9.15 per cent; total organic extractives 1.43 and 1.41 per cent; and inorganic ash 0.68 and 0.62 per cent.

This composition is characterized by its low content of salts and extractives and high content of protein. The protein is at least 70 per cent higher than in the yolk of the hen's egg. On the other hand the lecithins and neutral fats are less than half the amount stored in the yolk of the hen's egg. In the preincubation stage the organic extractives are low, much lower than in the muscle of the same fish.

*The Developing Ovaries.*

The ovaries contain ovarian tissue and developing ovules, the ovules with their stored food yolk forming an ever increasing proportion of the mass. This fact doubtless accounts for the slighter variations of composition as development progresses. The changes during development are presented under subtopics describing the various group constituents.

*The Water of the Ovaries.*

The water of the developing ovary is greatest in the mature ovary. The averages for each station are 54.08, 54.21, 52.05, 54.36, and 58.27 per cent. The increase at the spawning grounds coincides with the decreasing concentration of the blood observed in the king salmon of the Sacramento River.<sup>13</sup> It cannot be accounted for on the ground of loss of fat since the percentage of water on a fat-free basis is higher in spawning salmon.

<sup>13</sup>Greene, C. W., *Bull. U. S. Bureau Fisheries*, 1904, xxiv, 445.



*The Inorganic Salts.*

The total inorganic salts vary slightly but are a trifle higher in mature ovaries, averaging 0.57 per cent in relatively young ovaries in salmon from the mouth of the Columbia in comparison with 0.72 per cent from mature ovaries and in ripe ova. This variation is of little significance except that it gives one more confirmation of the fact that the saline content of salmon tissues is independent of the saline content of the water in which the salmon lives.

*The Proteins.*

The proteins of the developing ovaries differ little in amount from the content of ripe eggs. The station averages are: 25.62, 25.99, 25.71, 27.08, 26.10 per cent for the five stations, a variation of only 1.46 per cent. The highest protein observed was at the Ontario station and the lowest on the spawning grounds. The average for ripe eggs is 26.7 per cent which is nearly 11 per cent greater than the 16 per cent of protein present in the yolk of the hen's egg. The proteins form no inconsiderable part of the stored food of the salmon eggs.

*The Organic Extractives.*

The organic extractives of the ovaries are never great in amount. The averages are 1.55, 1.62, 1.63, 1.39, and 1.45 per cent from the five stations. The carbohydrates constitute 0.09 per cent of the extractives<sup>14</sup> and the remainder is assumed to be nitrogenous extractives. The average amount decreases slightly but not greater than the variation in duplicate determination. On the whole the ovarian extractives seem to obey the law of constant level of saturation as given for the nitrogenous extractives of muscle.<sup>15</sup>

The maintenance of the high level of protein during the growth in mass of the ovaries can only occur by the synthesis of proteins from the amino-acids of the blood and ovarian waters. The degree of saturation of the muscle waters by organic extractives amounts to 3.8 per cent, the ovarian waters are saturated only

<sup>14</sup>Greene, C. W., The glucose of salmon tissues, in press.

<sup>15</sup>Greene, C. H., *J. Biol. Chem.*, 1919, xxxix, 457.

to 2.5 per cent. While the muscle protein is being hydrolyzed protein is in process of synthesis in the developing ovaries. The loss of muscle proteins during the migration is more than enough to account for the gain in the proteins stored in the ovaries, a point supported by Paton in 1898. However, my view of the process is that the ovarian proteins are resynthesized from the hydrolytic products arising from the muscle proteins.

### *The Neutral Fats.*

The neutral fats represent the chief store of energy in the salmon's egg, just as the "egg oils" serve that function in the yolk of the hen's egg. The youngest ovaries carry the greatest amount of fats. The amounts at the three lower river fishing stations are 14.2, 13.5, and 17.4 per cent. The individual extremes are 13.3 and 18.2 per cent. Ontario salmon average 13.6 per cent with 10.3 and 15.1 per cent for the extremes. The average from the spawning grounds is 10.6 per cent. The lowest fat found was 9.1 per cent in the ripe eggs of Salmon 1,299.

The series shows a great variation in stored fats of the ovaries, just as was found for the muscles. But the spawning fish have a decidedly lower content of fat. This fact argues for the dependence of the ovarian fats on the general fat stores and on the lipolytic processes of the body of the salmon. The ovaries are not depleted of their fats to the low level of the fats of muscle, but do yield from 30 to 40 per cent of the store of fats present in the younger stages.

As with the proteins so with the egg oils, the storage takes place from the lipins liberated from supplies in the muscles, liver, connective tissues, etc. The reversible action of the lipases is adequate to account both for the storage of ovarian fats and for their percentage decrease as the fats are depleted from the body by oxidation during the migration.

### *The Phospholipins.*

The phospholipins represent the most complex food product stored in the developing ovaries. In the yolk of the hen's egg the phospholipins are present to the extent of 11 per cent and more along with about twice that amount of neutral fats. But in the

salmon ovary the amount is far less, from 2 to 3.6 per cent of the mature eggs.

The phospholipins of the ovaries from the three stations on the lower Columbia amount to 4.1, 4, and 3.4 per cent. At Ontario the average has dropped to 2.9 per cent, although one ovary contained as much as 4 per cent of phospholipins. At the spawning grounds the average phospholipin content is 2.6 per cent, with 1.9 and 3.6 per cent as the extremes.

The determinations given for the phospholipins represent the greatest variations in the data. The variations bear no direct relation to the neutral fats present in individual ovaries. However, like the neutral fats, the phospholipins decrease in amount with approaching maturity of the ova. The 4 per cent average for an ovary of 500 gm. weight drops to an average of 2.6 per cent for mature ovaries weighing 700 to 2,500 gm.

The significance of this decrease and variability is somewhat a matter of conjecture. It was argued by Paton<sup>16</sup> that the lecithins of the muscle are not adequate to provide the store found in the mature ovaries of the Scottish salmon, but loss of muscle phosphorus was equal to gain of ovarian lecithin phosphorus. The muscles of the king salmon have an average of 1.18 per cent of phospholipins when they begin the migration.<sup>1</sup> This drops to 0.44 per cent for spawning salmon. This loss, considering the large mass of the muscle tissue, is much greater than the gain to the developing ovaries. However, in view of the more recent advances in knowledge of lecithin metabolism<sup>17</sup> it is not to be assumed that the muscle lecithins are transported unchanged from muscle to ovary. It is more plausible to assume that these phospholipins are resynthesized in the ovary from available fats and phosphorus rests coming to the organs in the blood. It would be indeed difficult to prove this contention by direct tests on the salmon itself, a fact the writer keenly realizes.

On the whole it seems evident that the phospholipins play a less dominant rôle, at least they form a far less proportionate amount of the stored food materials, in the salmon ovaries and eggs than they play in the case of the hen's egg. The fats, too,

<sup>16</sup> Paton, D. N., *Rep. Fishery Bd. Scotland*, 1898, iv, 143.

<sup>17</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577. This paper contains a full reference list.

form a smaller percentage of the food store of the egg. The dependence of the salmon on fats and fatty bodies for energy during the migration is reflected in the partial depletion of both neutral fats and phospholipins in the mature salmon eggs. The proteins are present in unusual amount and doubtless play a leading part in the nutrition of the developing embryo.



# A CHEMICAL STUDY OF CERTAIN PACIFIC COAST FISHES.\*

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## INTRODUCTION.

In reviewing the literature on the chemical composition of fish, it is noticed that very few species have been the subject of extended investigations. The first extensive record of analyses of fish is that of Atwater published in 1888 (1). His notable investigations covered fifty-two American species, chiefly of Atlantic source but including some from the Pacific. Of his analyses, twenty-seven were based on one sample, thirteen on two samples, and twelve on from three to seven samples. Varying numbers of individuals entered into the composition of each sample. He examined nine species at different seasons of the year, the results in the case of three of these indicating seasonal variation. His figures on salmon also clearly indicate a loss in fat content during the spawning migration.

A second noteworthy investigation was conducted by Clark and Almy (2). They made a series of analyses of Atlantic Coast fishes during 1915 in order to obtain additional data on the seasonal variation both in the proximate composition and in the physical and chemical fat constants. Most of their samples were of a composite nature based on an average of three or four fish.

\* The work presented here was begun in May, 1918, under the direction of Dr. E. D. Clark and continued under Mr. A. W. Hansen until July, 1919. Since that time until June, 1920, it was carried on under the supervision of Dr. C. L. Alsberg, Chief of the Bureau. The analyses were begun at Stanford University, continued in the laboratory of the National Cannery Association at San Pedro, California, and completed at the San Diego branch of the Food Research Laboratory under the supervision of Dr. L. H. Almy.

One series was completed in the spring of 1915 and the other in the fall of the same year. They analyzed the shad before and after spawning. They also analyzed four composite samples of three fish each from the same school of weakfish. To summarize their findings briefly, they found considerable variation in many species (bluefish, butterfish, carp sucker, and weakfish) from spring to fall; the shad loses greatly in fat content during spawning; weakfish of the same school, caught at the same time, may show a wide variation in fat content (four composite samples of three fish each showed fat percentages of 1.35, 2.47, 4.88, and 8.03).

Many studies have been made of the changing composition of the king salmon during their fasting migration to the spawning grounds. In a recent publication, Greene (3) shows that spawning king salmon which have been in fresh water without eating at an estimated time of from 4 to 5 months have a fat content of 2.63 per cent and a protein content of 13.71 per cent contrasted with 16.43 per cent fat and 16.97 per cent protein in tide-water fish. Greene concludes that the king salmon stores up both fat and protein for its spawning migration.

It has frequently been pointed out that there is a relation between sea temperature and fat content of fishes. Murray and Hjort (4) stated that the fat contents of the sprat which abounds off the coast of Norway increase during summer when there is a rise in sea temperature, while both decrease toward the end of the year; it is concluded that the growth of the fish must be influenced by the prevailing temperatures in different waters.

Perhaps the most detailed investigation of the proximate composition of a selected species has been made by Johnstone (5). He selected the herring as a subject and his analyses were made of fish caught during the years 1914, 1916, and 1917. His samples were always composite ones usually based on ten fish, five males and five females. Most of the fish he analyzed were mature although he does not usually make mention of the size in connection with the analysis. However, he has made a careful record of the degree of development of the gonads in every case and hence has obtained an exact record of the relation of the chemical composition of both sexes to the sexual cycle.

In this report Johnstone remarks:

"In all races of herrings the maturation of the gonads is accompanied by an increase of fat in the flesh. For some time before the fish spawns (but after the major part of increase in the mass of the gonads has taken place) the fat contents decrease, and after spawning this decrease becomes very rapid. Between the time of spawning and the time at which maturation of the gonads begins again, the fat content of the flesh is at its minimum value."

#### *Procedure.*

The primary objects of our investigation were to study the seasonal variation in several species of fish and to obtain data from which food values can be ascertained. Many of the earlier analyses, based on but a few fish and made at only two or three widely separated dates, have been omitted and this report has been confined to two subjects; the variation in composition of individual fish, and the seasonal variation in the composition of the mackerel and mackerel-like fishes.

The fish used for analysis were obtained from the wholesale fish markets, from boats, or from canneries. From five to ten fish were usually selected for analysis. When the fish were small, all of the flesh was removed from one side of each, scales and bones were separated, and the remaining edible portion ground three times in a meat chopper. A sufficient quantity of the sample thus obtained was kept in a stoppered flask until the analysis was completed. In the case of larger fish, sections 1 inch or more in thickness were taken from one side of each fish and prepared for analysis as above.

Ether extract and ash were determined by the official methods.<sup>1</sup> Total solids were found by weighing out 10 gm. of the sample into a lead dish which contained a small quantity of ignited sand. After drying to constant weight in a water bath oven at 98-99°, the same sample was used for the determination of ether extract. Total nitrogen was determined by the Kjeldahl and Gunning method. The results in all cases represent the average of two closely agreeing determinations.

#### *Variation in the Composition of Individual Fish.*

Evidence that analyses based on but a few fish may lead to incorrect conclusions has been found in studies of several species.

<sup>1</sup> Bull. 107 (revised), Bureau of Chemistry.



Those which have been investigated and which show marked and often erratic difference in composition are yellow fin tuna, blue fin tuna, sable-fish, barracuda, mackerel, and sardine. Data on the last named species will be presented in another paper.

In September, 1920, six yellow fin tuna (*Germo macropterus*) each weighing between 25 and 30 pounds were found to have fat percentages of 5.84, 5.71, 4.88, 2.65, 1.82, and 0.20. These six fish were selected at random from a large catch. The average fat content is 3.52 per cent although the first three fish average 5.48 per cent and the last three 1.56 per cent, a striking variation.

In September, 1918, five individual blue fin tuna showed fat percentages of 7.95, 8.71, 9.39, 10.04, and 10.76. These fish were of about the same size and were from the same boat-load. Although the variation in fat content is not striking yet the figures are valuable in showing a certain individual range in composition.

It would seem to be a natural inference that immature fish should show a lower fat content than mature fish of the same species. Some data in agreement with this inference has been found. Thus an immature sable-fish (*Anaplopoma affinis*) caught April 4, 1918, had a fat content of 0.07 per cent while a mature sable-fish, caught 2 months later, had 14.87 per cent fat. These results are in accordance with the expectation and may be characteristic of this species.

That this condition does not hold for all species was found when large, medium, and small barracuda were analyzed. Three analyses were made of this species (*Sphyræna argentea*). The first sample, consisting of ten fish caught off the coast of Lower California in December, 1918, weighing about 5 pounds apiece, had 1.85 per cent fat. The second sample, from ten fish caught at the same time and place and weighing about 2 pounds apiece, contained 6.45 per cent fat. The third, based on fish caught off San Pedro a month later, averaging 0.5 pound apiece had a fat content of 1.51 per cent.

On several occasions a number of individual mackerel from the same catch have been analyzed. These analyses, as shown in Table I, are arranged in several chronological series and the fish in each series are in an increasing order as regards weight. In the series of October 25, 1918, variation from a minimum of 0.85

per cent fat to a maximum of 7.88 per cent was found although the fish were of nearly the same size. About a month later a sample from six males had about the same composition as

TABLE I.

*Variation in the Composition of Individual Mackerel (Scomber japonicus).*

No.	No. analyzed.	Description.	Average weight.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N X 6.25).
			gm.		per cent	per cent	per cent	per cent	per cent
1	1		496	Oct. 25, 1918	25.92	1.27	1.27	3.76	23.50
2	1		526	" 25, 1918	26.20	0.85	1.52	3.89	24.31
3	1		546	" 25, 1918	31.71	7.88	1.34	3.66	22.88
4	6	Males.	511	Nov. 17, 1918	24.35	0.41	1.41	3.69	23.06
5	6	Females.	511	" 17, 1918	24.89	0.85	1.37	3.74	23.37
6	1	Male spent.	582	Aug. 7, 1919	28.16	3.50	1.45	3.77	23.56
7	1	Female spent.	596	" 7, 1919	26.83	2.91	1.47	3.65	22.81
8	1	" "	596	" 7, 1919	28.03	3.87	1.47	3.69	23.06
9	1	" "	624	" 7, 1919	28.18	4.48	1.44	3.49	21.81
10	1	" full.	823	" 7, 1919	32.65	9.81	1.32	3.43	21.44
11	1	" "	1,135	" 11, 1919	30.36	6.35	1.40	3.69	23.06
12	9	Males "	1,220	" 11, 1919	30.69	7.50	1.31	3.50	21.87
13	1	Female.	538	Nov. 18, 1919	28.57	5.25	1.56	3.58	22.38
14	1	"	568	" 18, 1919	32.57	9.78	1.43	3.50	21.88
15	1	"	568	" 18, 1919	32.31	9.34	1.23	3.55	22.19
16	1	"	625	" 18, 1919	34.21	11.68	1.35	3.66	22.88
17	1	"	682	" 18, 1919	32.96	10.69	1.37	3.53	22.06
18	1	Male.	1,022	" 18, 1919	29.33	6.86	1.33	3.47	21.68
19	1	"	1,079	" 18, 1919	37.67	15.45	1.28	3.33	20.81
20	1	Female.	1,193	" 18, 1919	37.25	15.84	1.20	3.27	20.44
21	1	"	1,332	" 18, 1919	39.84	18.93	1.04	3.25	20.31
22	1	Male.	1,333	" 18, 1919	41.05	20.32	1.04	3.27	20.44
23	1		538	Dec. 10, 1919	30.65	8.41	1.23	3.52	22.00
24	1		625	" 10, 1919	33.00	10.32	1.31	3.45	21.56
25	1		681	" 10, 1919	33.99	12.35	1.35	3.45	21.56
26	1		838	" 10, 1919	37.48	17.78	1.34	3.40	21.25
27	1		938	" 10, 1919	30.31	7.77	1.52	3.45	21.56

one based on six females, both having less than 1 per cent fat. Five mackerel analyzed August 7, 1919, had fat contents increasing in much the same order as their increasing weights.

The same relation was found November 18, 1919, with the exception of Sample 18 which had a proportionately low fat content. On December 10, 1919, Nos. 23 to 26 showed close relationships between size and fat contents, while No. 27, the largest of the series, had the lowest fat content.

The data given in Table I indicate that fat content may vary widely in mackerel of the same catch and of about the same size (Nos. 1 to 3); sex appears to bear little if any relation to proximate chemical composition; although the percentage of fat generally increases with the size, Nos. 3, 18, and 27 are marked exceptions to this rule; finally, mackerel of the same size may have different fat contents at corresponding times of consecutive seasons (Nos. 1 to 5 contrasted with Nos. 13 to 17). As some of the mackerel analyzed in August, 1919, were spent (Nos. 6 to 9) and some were full (Nos. 10 to 12), it is evident that the spawning time of this species is midsummer.

#### *Seasonal Variation in the Composition of the Mackerel-Like Fishes.*

Of the various mackerel-like fishes, the species which is available throughout the year and most easily secured in the wholesale markets is the California mackerel (*Scomber japonicus*). Accordingly, most of the analyses of mackerel-like fishes have been confined to this species. As has been shown, there is considerable variation in individual mackerel and hence the samples analyzed, with the exception of a few of the earlier ones, are based on not less than five fish. It will be seen by referring to Table II that in many cases five small and five large fish of the same catch were segregated and the two composite samples analyzed separately. This was not always possible because the larger sized fish often could not be obtained. With the exception of Samples 1, 4, 5, and 6, obtained from Monterey Bay, all of the samples were taken off southern California and brought in to San Pedro. Samples 4, 5, and 6 were analyzed by Mr. A. W. Hansen of the Bureau of Chemistry.

It will be seen that of the samples averaging more than 1 kilo in weight, the two highest in fat content, Nos. 12 and 17, were in October, 1919, and February, 1920; the next highest was in August, 1919 (No. 9); while the lowest, No. 21, was in May, 1920. It has already been pointed out from the data shown in

Table I that small mackerel analyzed individually in the fall of 1918 at San Pedro were much lower in fat content than those of the succeeding fall. Samples 2 and 3 contrasted with Nos. 10 and 11 (Table II) further illustrate this fact. Considering mackerel caught during 1918-20 of from 375 to 680 gm. in weight, it is noticed that the sample of lowest fat content, No. 7, was

TABLE II.  
*Analyses of the California Mackerel (Scomber japonicus).*

No.	No. analyzed.	Description.	Average weight.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N×6.25)
			gm.		per cent	per cent	per cent	per cent	per cent
1	1		400	June 3, 1918	27.17	3.62	1.27	3.50	21.87
2	3		523	Oct. 25, 1918	27.94	3.33	1.38	3.77	23.57
3	12		511	Nov. 17, 1918	24.62	0.63	1.39	3.71	23.19
4	2		505	" 1, 1918	28.61	4.64	1.46	3.58	22.37
5	4		503	" 25, 1918	25.60	1.51	1.41	3.56	22.25
6	5		348	Dec. 19, 1918	28.08	4.69	1.25	3.53	22.06
7	10	Filling.	500	May 12, 1919	23.38	0.28	1.47	3.55	22.19
8	5	1 full; 4 spent.	644	Aug. 7, 1919	28.77	4.91	1.43	3.61	22.56
9	9	All full.	1,220	" 11, 1919	30.69	7.50	1.31	3.50	21.87
10	10	Spent.	680	Oct. 9, 1919	33.83	10.16	1.35	3.68	23.00
11	5	"	450	" 20, 1919	31.69	8.49	1.37	3.61	22.56
12	5	"	1,350	" 20, 1919	38.80	18.12	1.31	3.27	20.44
13	10	Virgin.	894	Nov. 18, 1919	34.58	12.41	1.28	3.44	21.50
14	5	Filling.	724	Dec. 10, 1919	33.09	11.32	1.35	3.45	21.56
15	10	"	375	Jan. 19, 1920	27.59	4.70	1.41	3.57	22.31
16	5	"	551	Feb. 12, 1920	26.52	3.65	1.50	3.61	22.56
17	5	"	1,290	" 12, 1920	38.63	18.08	1.24	3.16	19.75
18	5	"	510	Apr. 7, 1920	27.46	4.38	1.41	3.60	22.50
19	5	"	760	" 7, 1920	30.53	7.49	1.42	3.52	22.00
20	5	"	666	May 29, 1920	25.39	1.02	1.43	3.72	23.25
21	5	"	1,370	" 29, 1920	26.97	3.45	1.38	3.59	22.44

caught in May, 1919, while No. 3, caught in November, 1918, and No. 20, caught in May, 1920 were nearly as low in percentage of fat. The two of maximum fat content are Nos. 10 and 11, caught in October, 1919. Nos. 15, 16, and 18, caught in January, February, and April, 1920, respectively, had an intermediate fat content.

We are therefore justified in concluding that the mackerel undergoes a seasonal variation in composition; that large mackerel are generally fatter than small mackerel of the same school, and that the variation in one season may not be paralleled by the next season's variation. The period of maximum fat content during 1919 followed the spawning season, while the period of minimum fat content preceded the spawning season. In the previous season the percentage of fat appeared to be at a minimum in the late fall after the spawning season.

Several analyses have been made of the other common mackerel-like fishes of southern California. With the exception of the bonita, none of the species whose analyses are shown are commonly taken in a spawning condition on this coast. It is generally considered that the blue fin tuna and albacore of the size usually taken, from 16 to 25 pounds in weight, are immature fish. For this reason the only analyses of large tuna or albacore, Nos. 4 and 5, should be considered in a class by themselves. They are quite likely mature spent fish. Such fish are not commonly taken off southern California until late in the fall. Whether such species migrate great distances, or whether they seek deep water for spawning, is as yet a mystery.

The analyses of the fish shown in Table III are too limited in number to give satisfactory information as to seasonal variation. It is apparent that considerable fat is stored in the flesh of these species during June, July, August, and September. In considering these analyses, it should be borne in mind that they are based on flesh only. The skin and heads frequently have a relatively high fat content when the amount of fat in the flesh is small. Thus, on one occasion it was found that a sample of skins, heads, and bones of albacore, even after the loss of some of their fat by cooking, had a fat content of 11.16 per cent while the per cent of fat in the flesh of the same cooked fish was 4.91. Before cooking the flesh had a fat content of 5.22 per cent (Sample 1).

#### CONCLUSIONS.

Large variations in the composition of individual fish of several species (yellow fin tuna, blue fin tuna, sable fish, barracuda, and mackerel) have been found. These variations are frequently erratic and cannot be ascribed to known factors.

TABLE III.  
*Analyses of Various Fishes of the Mackerel Family.*

No.	Species.	Average weight.	No. analyzed.	Date.	Composition of the edible portion.				
					Solids.	Ether extract.	Ash.	Total nitrogen.	Protein (N $\times$ 6.25).
		lb.			per cent	per cent	per cent	per cent	per cent
1	Albacore ( <i>Germo alalunga</i> ).....	20-30	5	June 19, 1919	32.05	5.22	1.34	4.11	25.69
2	" ".....	20-25	5	July 22, 1919	31.80	4.35	1.30	4.27	26.69
3	" ".....	20-25	5	Sept. 8, 1919	38.49	12.76	1.35	3.85	24.06
4	" ".....	40	5	Oct. 20, 1919	31.71	5.50	1.33	4.15	25.94
5	" ".....	50	5	Nov. 20, 1919	33.21	6.92	1.30	4.09	25.56
6	Blue fin tuna ( <i>Thunnus thynnus</i> ).....	23	5	Sept. 21, 1918	34.45	9.37	1.32	3.91	24.44
7	" ".....	20	5	July 22, 1919	27.43	1.08	1.43	4.02	25.13
8*	Yellow fin tuna ( <i>Germo macropterus</i> ).....	25	10	May 14, 1919	27.17	1.00	1.47	4.06	25.31
9	" ".....	30	5	Sept. 8, 1919	30.83	6.54	1.32	3.84	24.00
10	" ".....	14	3	" 15, 1920	29.27	3.29	—	4.03	25.19
11	" ".....	28	6	" 22, 1920	29.23	3.52	—	3.93	24.56
12*	" ".....	20	6	Oct. 25, 1920	26.05	0.90	—	3.89	24.31
13	Striped tuna ( <i>Gymnosarda pelamis</i> ).....	6-7	5	Aug. 18, 1919	33.48	6.62	1.28	4.08	25.50
14	" ".....	6-7	5	Oct. 23, 1919	34.64	8.11	1.32	4.05	25.31
15	Bonita ( <i>Sarda chilensis</i> ).....	9	1	Sept. 21, 1918	41.08	19.21	1.34	3.27	20.44
16	" ".....	6-7	6	May 19, 1919	26.26	1.21	1.47	3.97	24.81

\* Samples 8 and 12 were caught off Lower California. Others all from off southern California.

The variation in the composition of the mackerel during one season was not paralleled by the next season's variation.

The spawning season of the mackerel was found to be mid-summer. No evidence of a connection between decreasing fat content and the approach of the spawning season was found in the mackerel. On the contrary, the spawning season in 1919 appeared to come during a time of increasing fat content.

No evidence of a connection between the sex and the proximate chemical composition of the mackerel was found.

With some exceptions, the mackerel and the mackerel-like fishes were found to have an increasing fat content through the summer and early fall.

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## THE POTASSIUM CONTENT OF NORMAL AND SOME PATHOLOGICAL HUMAN BLOODS.\*

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Our interest in the potassium content of human blood was aroused some time ago by the observation of Smillie<sup>1</sup> that poisoning may result from the administration of potassium salts to certain nephritic patients. This was noted in a case of nephritis and later confirmed in experimental uranium nephritis. Smillie states:

"In human beings, potassium chlorid, in doses which have no effect on normal individuals, will cause acute poisoning in individuals with chronic nephritis.

"This acute poisoning occurs because the salt, which is normally readily absorbed and very rapidly excreted, in nephritis is readily absorbed and not excreted, thus reaching a concentration in the blood which is injurious."

Owing to the fact that in the human species potassium is an important constituent of the corpuscles and present in them in a much higher concentration than in the plasma, it is quite necessary that this factor should always be taken into account in any estimation of the potassium content of human blood. The composition of the blood of different species of animals was carefully considered by Abderhalden,<sup>2</sup> and Table I is recalculated from his data. As will be noted the potassium content of whole blood in the horse, pig, and rabbit, and also man (Tables II and III) stand in marked

\* A preliminary report of these observations was made before the Society for Experimental Biology and Medicine, November 17, 1920, Myers, V. C., and Short, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 72.

<sup>1</sup> Smillie, W. G., *Arch. Int. Med.*, 1915, xvi, 330.

<sup>2</sup> Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 106.



TABLE I.

*Potassium Content of the Blood of Different Species of Animals.\**

Species.	Potassium.	
	Whole blood.	Serum.
	<i>mg.</i>	<i>per 100 cc.</i>
Horse 1.....	227	22
“ 2.....	123	21
Pig.....	192	23
Rabbit.....	175	22
Ox.....	34	22
Bull.....	34	21
Sheep 1.....	34	22
“ 2.....	34	21
Goat.....	33	21
Cat.....	22	22
Dog 1.....	22	22
“ 2.....	21	19

\* Observations recalculated from Abderhalden.<sup>2</sup>

TABLE II.

*Potassium Content of Human Blood.\**

Case.	Diagnosis.	Age.	Sex.	Potassium.	
				Whole blood.	Serum.
				<i>mg.</i>	<i>per 100 cc.</i>
1	Normal.	25	♂	174	31
2	“	30	♀	161	33
3	Cholera.	26	♀	185	
4	“	55	♂	225	
5	“	20	♀	166	43
6	“	71	♂	203	72
7	“	23	♂	194	62
8	Diabetes.	34	♂	170	32
9	Chronic edema with albuminuria.	39	♂	116	21
10	Anasarca without albuminuria.	42	♂	190	63
11	Normal dog.			37	29

\* Observations recalculated from Schmidt.<sup>3</sup>

contrast with that found in such carnivorous animals as the cat and dog, where the findings for whole blood and serum are almost identical.

We have long possessed data on the potassium content of human blood as the result of the analyses carried out by Schmidt<sup>3</sup> in 1850. The data in Table II are recalculated from his analyses. The rather high findings for potassium in the whole blood of the cholera cases can probably be explained on the basis of the concentration of the blood found in this condition, but this would scarcely explain the high figures for the serum in two of these cases and one of the cases of nephritis. As has been pointed out by Macallum,<sup>4</sup> Schmidt's figures for the potassium content of the normal blood are likewise rather high.

Despite the fact that figures for the potassium of human blood were given by Schmidt in 1850, comparatively few data have since been recorded in the literature. A few analyses were reported by Macallum in 1917. He gives the normal potassium content of human blood plasma as 19 to 21 mg. per 100 cc., which, as will be noted, is about 60 per cent lower than the figures given by Schmidt for normal individuals. Regarding pathological cases Macallum states that his results obtained for the plasma in Bright's disease are quite incomplete but those for puerperal eclampsia are far enough advanced to furnish some points of interest. His potassium figures (four cases) are not given in absolute amount, but in relation to the sodium, taking the latter as 100. In these cases the ratio of the potassium to the sodium was increased two to four times. Since severe eclamptics generally suffer from quite pronounced acidosis, and sometimes from salt retention, it would seem logical to expect greater fluctuation in the sodium than in the potassium. On this account it is difficult to draw conclusions from changes in the ratio between the elements.

A few figures for the potassium content of blood have also been given by Drushel,<sup>5</sup> Greenwald,<sup>6</sup> Clausen,<sup>7</sup> Kramer,<sup>8</sup> and Kramer

<sup>3</sup> Schmidt, C., *Charakteristik der epidemischen Cholera gegenüber verwandten Transsudationsanomalien*, Leipsic and Mitau, 1850.

<sup>4</sup> Macallum, A. B., *Tr. College Phys. Philadelphia*, 1917, xxxix, series 3, 286

<sup>5</sup> Drushel, W. A., *Am. J. Sc.*, 1908, xxvi, 555.

<sup>6</sup> Greenwald, I., *J. Pharmacol. and Exp. Therap.*, 1918, xi, 281; *J. Biol. Chem.*, 1919, xxxviii, 439.

<sup>7</sup> Clausen, S. W., *J. Biol. Chem.*, 1918, xxxvi, 479.

<sup>8</sup> Kramer, B., *J. Biol. Chem.*, 1920, xli, 263.

and Tisdall.<sup>9</sup> Drushel obtained 166 mg. of potassium per 100 cc. in defibrinated pig's blood, 50 mg. in sheep's blood, 20 mg. in the serum of dog's blood, and 16 mg. in dog's lymph. Greenwald found the potassium content of dog's blood (serum and whole blood) to vary from 14 to 27 mg. per 100 cc., while Clausen found the potassium content of human whole blood (pathological cases) to vary from 143 to 290 mg. per 100 cc. and in plasma from 53 to 90 mg. In his first paper, Kramer gives the normal potassium content of human serum as varying between 16 to 22 mg., while in a more recent paper Kramer and Tisdall state that the potassium content of the serum of both normal children and adults is singularly constant, the maximum variation being from 18 to 21 mg. per 100 cc. In a series of fifteen miscellaneous pathological conditions in children they report figures ranging from 23 to 70 mg. per 100 cc. of serum. The analyses reported by Kramer and Tisdall were made by a direct precipitation method without ashing. It seems rather difficult to understand why miscellaneous pathological conditions should show such a marked difference from the normal as is the case with the results of Clausen, and Kramer and Tisdall.

In 1909 Myers<sup>10</sup> carried out a study of the potassium content of the spinal fluid of insane patients. The interesting observation was made that the potassium content of spinal fluid increased very rapidly after death, as high figures being obtained one-half hour post mortem as at any later time. It was stated at that time that:

" . . . the potassium content of the cerebrospinal fluid during life corresponds very closely to the amount of potassium in the blood serum, while after death the quantity of potassium in the cerebrospinal fluid agrees more nearly with that of the whole blood."

The figures obtained for fifteen living cases varied from 14 to 28 mg. and averaged 22 mg. potassium per 100 cc. of spinal fluid. For the twenty-two specimens of spinal fluid obtained after death the findings ranging from 57 to 105 mg. with an average of 83 mg. per 100 cc.

<sup>9</sup> Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlv, 339.

<sup>10</sup> Myers, V. C., *J. Biol. Chem.*, 1909, vi, 115.

The estimations of Abderhalden and Schmidt previously referred to were carried out with the chloroplatinate method, but in most recent observations, the cobalti-nitrite method has been used. Drushel, Clausen, Kramer, and Kramer and Tisdall have described adaptations of this method to blood analysis. In the last mentioned method the potassium is precipitated directly from the serum without ashing. We have used the cobalti-nitrite method of Drushel,<sup>5,11</sup> essentially as it was employed by one of us for the spinal fluid more than 12 years ago.

Our experience would lead us to believe that more satisfactory results are obtained on serum than on plasma, owing to the fact that hemolysis is more likely to take place when sodium citrate or ammonium oxalate have been added. Since human whole blood contains about ten times as much potassium as the serum it is essential to guard against the passage of any potassium from the cells. In most of our analyses the corpuscles have been separated from the serum by a double centrifuging about 2 hours after the specimens have been taken. Furthermore, when there has been any question about hemolysis, the serum has been subjected to spectroscopic examination for absorption bands of oxyhemoglobin. Slight hemolysis, however, does not necessarily greatly increase the potassium content of the serum. The potassium estimations on whole blood have been made on blood to which (potassium-free) sodium citrate was added as the anticoagulant. The analytical procedures we have employed are described below.

### *Method.*

5 cc. of blood serum or 1 cc. of whole blood are treated in a 125 cc. platinum evaporating dish with 5 cc. of a 1 to 10 sulfuric-nitric acid mixture and evaporated down rapidly in a hood over a low Bunsen burner flame. When the mixture reaches a small volume and begins to char, nitric acid is added, a few drops at a time, and the heating continued until it foams up. At this point the dish is covered with small ashless filter paper to prevent loss of material from spattering during the final steps in the oxidation. After the material has become nearly dry from the low heat, the flame is turned on full until all the organic material, including the filter paper, is burned up, and the substance is completely ashed.

The ash is now dissolved in 3 to 5 cc. of hot water, 1 to 2 cc. of glacial acetic acid added, and then transferred to a 50 cc. beaker with the aid of

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<sup>11</sup> Drushel, W. A., *Am. J. Sc.*, 1907, xxiv, 433.

TABLE III.  
*The Potassium Content of Normal and Some Pathological Human Bloods.*

Case.	Age.	Sex.	Potassium.		Total solids.	Red cells.	Chlorides as NaCl.	Other blood analyses.				Clinical diagnosis.
			Serum.	Whole blood.				Uric acid.	Urea N.	Creatinine.	Sugar.	
			mg. per 100 cc.	mg. per 100 cc.	per cent	millions	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	
1. F. W.	32	♂	14	158	19.3							Normal.
2. G. B.	26	♂	18	269	21.6							"
3. L. S.	20	♂	18	195	21.4							"
4. C. W.	26	♂	18	141								Chronic diffuse nephritis, died.
5. M. D.	26	♂	10	52	13.4		537	5.1	43	5.5	0.121	52
6. M. B.	41	♀	15	134	14.7	3.1	531	7.0	102	8.3	0.159	33
7. S. T.	39	♂	17	88	13.3	3.6	594	9.3	100	12.0	0.136	20
8. T. D.	50	♀	18		13.3	3.1		6.5	95	5.7	0.142	29
9. M. W.	30	♂	19	94	11.7	4.0	444	9.7	165	13.1	0.160	26
10. J. S.	17	♂	16	96	15.6	4.0	507		53	3.6	0.150	51
11. B. G.	27	♂	20	94	14.2	2.2	435		115	16.0	0.163	
12. C. C.	32	♀	28*	59	12.0	1.7	469	18.4	214	25.0	0.328	52
13. A. B.	32	♀	28†	148	18.7	4.1	531	4.3	21	3.0	0.121	

14. F. M.	57	♂	35†	81	15.7	2.1	568		59	4.8	0.153		Double polycystic kidney, secondary anemia.
15. H. K.	30	♂	20	146			519		12	2.2	0.145		Cardiovascular disease.
16. C. O'N.	52	♀	20				494		10				Cardiac decompensation, ana- sarca.
17. J. S.	41	♂	15			4.5	488	3.1	19		0.143		Essential hypertension.
18. S. D.	50	♀	28†	168	21.0		475		11	2.2	0.101		"
19. M. C.	47	♂	16			4.9	450	5.1	25	2.2	0.145		Carcinoma of esophagus.
20. J. S.	47	♂	16				488		18		0.143		Lobar pneumonia, conva- lescent.
21. R. W.	24	♀	17				550		7	2.5	0.130	40	Eclampsia.
22. F. W.	55	♀	24†						18	2.3	0.510	21	Diabetic coma, died.
23. A. B.	50	♀		124	19.3	3.5							Pernicious anemia.
24. M. T.	46	♀	18	72		1.8							Syphilis, pernicious anemia (?).

\* Blood taken from heart about 45 minutes after death.

† Specimens taken before the necessity for the almost immediate separation of serum from the clot was fully appreciated.

a rubber-tipped rod. 10 cc. of the sodium cobalti-nitrite reagent are added, the beaker is covered with a watch-glass and placed in the refrigerator at 4°C. over night. On the following day the contents of the beaker are filtered through a porcelain Gooch crucible with thick asbestos mat, and washed with about 100 cc. of ice cold water.

The asbestos mat is now removed with a stirring rod and placed in a beaker containing 10 cc. of 0.1 N potassium permanganate and about 100 cc. of distilled water nearly at the boiling point and stirred. The crucible is then immersed in the hot permanganate solution in order to oxidize the last trace of the precipitate which may have adhered to it. The solution is now heated for 5 or 6 minutes until manganese hydroxide begins to separate out and the solution darkens. 10 to 15 cc. of 1 to 7 sulfuric acid are next added, and the solution, after stirring, is allowed to stand for several minutes. A known excess of 0.1 N oxalic acid (generally 10 cc.), containing 50 cc. of strong sulfuric acid to the liter, is then run into the beaker. After the permanganate color is thoroughly bleached, the crucible is removed, washing it with hot water. The hot solution is now titrated to color with the permanganate, the excess of permanganate over the oxalic representing the amount of potassium. After the titration the asbestos is again transferred to the Gooch crucible, washed with hot water, and the crucible set aside for the next determination.

Drushel has calculated that 1 cc. of 0.1 N potassium permanganate is required to oxidize 0.707 mg. of potassium in the form of  $K_2NaCo(NO_2)_6 \cdot H_2O$ . We have been able to use this factor in a large number of potassium estimations we have made on urine, feces, and muscle, but where one is working with very small amounts of potassium, as in the case of blood, it has generally been found desirable to employ an empirical factor. This was done by Adie and Wood<sup>12</sup> and by Myers<sup>10</sup> for spinal fluid. Such a factor has been worked out with each cobalti-nitrite solution for the amount of potassium determined (generally 1 mg.) under the conditions employed. Analyses have been made in duplicate.

Our observations are given in Table III.

#### DISCUSSION.

No evidence of a retention of potassium was found in the serum or whole blood of the seven cases of advanced nephritis given in Table III; all of these cases showed marked nitrogen retention. This does not exclude the possibility, however, that potassium retention may occur in cases with edema and marked chloride retention but without nitrogen retention. It may be noted that in Case 7, which showed considerable chloride as well as

<sup>12</sup> Adie, R. H., and Wood, T. B., *J. Chem. Soc.*, 1900, lxxvii, 1076.

nitrogen retention, the serum contained a normal amount of potassium.

According to a few original observations reported and others cited by Blumenfeldt<sup>13</sup> it appears that in certain cardiac and renal conditions there may be a retention of potassium with an increase of this element in the tissues. In view of our findings, and considering the fact that potassium is chiefly a constituent of body tissues rather than of body fluids, a comparison of the potassium content of the corpuscles, or of the whole blood on the basis of its hemoglobin content, would be more likely to disclose pathological variations than the method employed. It may be noted here that observations on the potassium content of the muscle tissue of the rabbit<sup>14</sup> have shown that starvation, or diets low in potassium, may reduce the potassium concentration of the muscle as much as 40 per cent.

As will be noted, none of our pathological cases appear to disclose any increase in the potassium content of the serum. In this respect our findings differ from the reported figures of Clausen, and Kramer and Tisdall.

It has recently been pointed out by Van Slyke and Cullen,<sup>15</sup> and Fridericia<sup>16</sup> that a shifting of the chlorides and CO<sub>2</sub> takes place when plasma is allowed to stand in contact with the cells, the chlorides of the plasma generally being increased. Since chloride estimations in the plasma are of value only when the plasma is separated as soon as the blood is drawn, Myers and Short<sup>17</sup> have recommended the estimation of the chlorides on whole blood. If immediate separation of the plasma from the corpuscles is necessary to prevent a change (increase) in the plasma chlorides, even though the chloride content of the cells is below that of the plasma, it is logical to believe that a rapid separation of the serum (or plasma) might be even more necessary in the case of the potassium, owing to the fact that the cells contain roughly twenty times as much potassium as the plasma. It is evident from the observations previously reported on spinal fluid that the permeability of certain cell membranes for potassium may change very rapidly after death.

<sup>13</sup> Blumenfeldt, E., *Z. exp. Path. u. Therap.*, 1913, xii, 523.

<sup>14</sup> Myers, V. C., unpublished observations.

<sup>15</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

<sup>16</sup> Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.

<sup>17</sup> Myers, V. C., and Short, J. J., *J. Biol. Chem.*, 1920, xliv, 47.



## CONCLUSIONS.

The potassium content of normal human blood serum amounts to rather less than 20 mg. per 100 cc., while for whole blood the figures are eight to twelve times this amount.

Owing to the high content of potassium in the cells, precautions should be taken in the analysis of serum or plasma to make the separation before any transfer of the potassium from the cells has taken place. We believe that the serum is preferable to the plasma for this determination. The separation of the serum should be made as soon as possible after the blood is withdrawn.

The potassium content of whole blood is roughly proportional to the total solid and red cell content.

In our series of seven nephritics with marked nitrogen retention no increase in the potassium content of the serum or whole blood was noted. On the contrary, the potassium content of the whole blood was diminished, apparently due in large part to an associated secondary anemia. These few observations do not lend support to the suggestion of Smillie that some of the symptoms of uremia may be due to a potassium poisoning as a result of retention of this element.

In none of our pathological cases were abnormal figures for the potassium of the serum found when the serum was separated within 2 hours after the blood was drawn.

# A CHEMICAL STUDY OF THE CALIFORNIA SARDINE (*SARDINIA CÆRULEA*).

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## INTRODUCTION.

The limited value of two or three analyses of each of a large number of species of fish has been emphasized in a previous paper. While making the study of Pacific Coast fishes it became apparent that it would be impossible to secure extensive information regarding the composition of more than a few species in the time allowed, and accordingly there was suggested the advisability of securing a large amount of data on a single species. The sardine was chosen because of its commercial importance, its availability, and the ease of sampling due to its small size. The methods of preparing and analyzing samples were the same as those described in the preceding paper.

At San Pedro, although sardines are packed every month in the year, most of the pack is put up from December to April. Sardines of commercial importance vary in weight from 15 to 250 gm. and are generally roughly divided into four groups. The smallest sardines, spoken of as "quarters" are packed in rectangular cans of about  $\frac{1}{4}$  pound capacity. Sardines of the next larger size, "halves," are generally packed in cans of  $\frac{1}{2}$  pound capacity. The third size is packed in oval cans of nearly 1 pound capacity which hold from seven to ten fish of this group. These sardines are called "small ovals." The fourth and largest size, "large ovals," is packed in the same can as the third but only four or five fish are required to fill the can. If one bears in mind that nearly one-half of the fish is removed during preparation for packing, a fair idea of the size of the fish in each group can be had.

The relative abundance of the different sizes varies in different seasons and in different localities. As a rule, "ovals" appear during December off southern California and disappear in June. During the first 6 months of 1919, "quarters" and "halves" were fairly abundant at San Pedro while few were to be had at San Diego. During the corresponding period of 1920, the reverse was the case; in fact it became necessary to discontinue the analyses of the small sardines because of the lack of samples.

As regards maturity, few if any of the smallest size are mature, while some of the second size, most of the third size, and all of the fourth size are mature. It will be seen then that a sardine which is mature so far as reproductive ability is concerned is not necessarily full grown.

#### *Results of Analyses.*

Unless otherwise stated, all of the sardine analyses have been based on composite samples of ten fish each. As will be noted in Tables I and II there is a wide variation in the composition of sardines depending in part on the size, maturity, and season. In order to find what individual differences might be expected at a time when the fat content is low, ten individuals of about the same size were analyzed separately on April 21, 1919. The results are given in Table III.

Since sardines of the two smaller sizes are generally immature, it appeared that the segregation of data on these two sizes might give information on the variation in composition as related to factors other than those connected with spawning. The monthly averages of the analyses of sardines in these two groups are shown in Table I. In Tables II and IV are shown similar analyses of "small ovals" and "large ovals" respectively.

Beginning in January, 1920, from six to nine samples of ten fish each were obtained about every 10 days. All of the fish of a given sample were of approximately the same weight and the average weight of the samples ranged from 80 or 100 gm. to 220 or 240 gm. in 20 gm. intervals. During this period, in preparing the samples for analysis, the gonads were carefully segregated and their degree of development ascertained by finding the ratio of their weight to the weight of the whole fish from which they were derived. The analyses which were made during the transition period from high

to low fat content are shown in Table V. The analyses of March 29, shown in this table, were from a school of fish which were still rather fat, while 10 days later and also 14 days later fish of quite different composition were found.

TABLE I.  
*Monthly Average Composition of Small Sardines.*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N $\times$ 6.25).
"Quarters." Average weight 15 to 40 gm.						
1918	gm.		per cent	per cent	per cent	per cent
Oct.....	30.4	1	22.80	0.48	3.31	20.69
Nov.....						
Dec.....	20.1	2	23.64	1.58	3.26	20.37
1919						
Jan.....	34.1	2	21.25	0.70	3.15	19.68
Feb.....	24.3	4	20.78	0.52	3.07	19.19
Mar.....						
Apr.....	32.1	2	22.98	1.06	3.34	20.88
May.....	28.2	2	23.57	1.47	3.32	20.75
June.....						
July.....	35.9	2	25.85	3.34	3.37	21.06
Aug.....	29.8	1	27.18	3.36	3.51	21.94
Sept.....	24.0	1	25.52	3.39	3.38	21.12
"Halves." Average weight 48 to 70 gm.						
1918						
Dec.....	54.6	2	26.05	3.05	3.34	20.88
1919						
Jan.....	61.8	2	23.67	3.11	3.13	19.56
Feb.....	51.0	1	16.88	0.34	2.41	15.06
Mar.....	48.1	1	23.44	2.66	3.06	19.12
Apr.....	60.5	2	23.42	3.21	3.21	20.06
May.....	69.0	1	20.21	0.39	3.04	19.00
June.....						
July.....	59.6	2	27.85	5.64	3.37	21.06
Aug.....	62.3	1	28.92	6.87	3.42	21.37

In studying the possible influence of development of gonads on the proximate composition of the flesh, several analyses of ovaries and testes were made; a record of the development of the gonads compared to the body weight was kept; and on two

TABLE II.  
*Monthly Average Composition of "Small Ovals."*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N X 6.25).
Average weight 80 to 120 gm.						
1918	gm.		per cent	per cent	per cent	per cent
Dec.....	105.3	3	26.93	5.38	3.18	19.87
1919						
Jan.....	103.2	4	30.25	9.62	3.10	19.37
Feb.....	85.8	2	23.27	2.95	3.08	19.25
Mar.....	99.1	1	28.88	7.44	3.20	20.00
Apr.....	85.5	2	20.65	0.77	3.11	19.09
May.....	93.7	2	21.31	0.60	3.11	19.09
Dec.....	86.5	2	32.25	12.06	3.03	18.93
1920						
Jan.....	100.0	3	31.68	10.74	3.17	19.81
Feb.....	104.0	5	32.12	11.66	3.09	19.31
Mar.....	104.0	10	30.17	9.49	3.12	19.15
Apr.....	105.0	8	26.20	4.50	3.21	20.06
May.....	113.0	3	26.20	3.69	3.40	21.25
June*.....	100.0	3	27.67	5.07	3.45	21.56

\* All June analyses were made June 3, as no sardines of this size could be obtained later in the month.

TABLE III.  
*Variation in the Composition of Individual Sardines.*

No.	Sex.	Average weight.	Composition of the edible portion.				
			Solids.	Ether extract.	Ash.	Total nitrogen	Protein (N X 6.25).
		gm.	per cent	per cent	per cent	per cent	per cent
1	Male.	162	20.92	0.52	1.59	3.03	18.94
2	"	170	20.89	0.24	1.63	3.09	19.31
3	"	165	19.07	0.13	1.80	2.87	17.94
4	Female.	184	25.92	4.66	1.46	3.23	20.19
5	"	172	21.43	0.39	1.68	3.07	19.19
6	"	162	22.09	0.80	1.47	3.11	19.44
7	"	160	21.71	0.09	1.59	3.21	20.06
8	"	162	24.14	1.80	1.57	3.36	21.00
9	"	152	20.12	0.10	1.66	3.07	19.19
10	"	162	20.31	0.13	1.57	2.99	18.69
Average .....		165	21.66	0.89	1.60	3.10	19.37

occasions ten relatively mature and ten relatively immature fish of each sex were segregated and analyzed. These results are shown in Tables VI, VII, and VIII, respectively.

TABLE IV.  
*Monthly Average Composition of "Large Ovals."*

Month.	Average weight per fish.	No. of analyses.	Composition of the edible portion.			
			Solids.	Ether extract.	Total nitrogen.	Protein (N× 6.25).
Average weight 140 to 260 gm.						
1919	gm.		per cent	per cent	per cent	per cent
Jan.....	225	4	37.71	19.20	2.79	17.44
Feb.....	186	2	33.34	14.02	2.88	18.00
Mar.....	225	3	35.41	15.83	2.89	18.06
Apr.....	188	2	21.08	0.75	3.03	18.93
May.....	191	5	23.27	2.74	3.11	19.07
June.....						
Dec.....	188	4	40.30	21.38	2.88	18.00
1920						
Jan.....	170	4	37.91	17.89	3.04	19.00
Feb.....	186	17	38.34	18.88	2.89	18.06
Mar.....	186	20	36.53	17.04	2.89	18.06
Apr.....	169	20	27.52	6.67	3.08	19.25
May.....	180	10	25.78	4.00	3.32	20.75
June*.....	170	4	25.22	2.75	3.38	21.13

\* All June analyses were made June 3, as no sardines of this size could be obtained later in the month.

#### DISCUSSION.

There may be a striking variation in the composition of individual sardines as will be seen from a study of Table III. Although the average fat content of the ten sardines analyzed was 0.89 per cent, eight of the ten fish had less than this amount while one, No. 8, had twice the average amount and another, No. 4, had over five times the average. Even when a composite sample is based on as many as ten fish, it has been found that erratic results may be obtained. There is usually a fairly regular increase in fat content with increasing size of the sardine. This increase is illustrated in the analyses of March 29 as shown in Table V which are typical

of fourteen similar series made from January to June, 1920. However, the analyses of April 8, shown in the same table, are quite variable due, no doubt, to the fact that there was great variation in individuals at this time just as there was during the same period of the previous season, as indicated in Table III.

It is apparent from the analyses of small sardines as presented in Table I that there is considerable although sometimes inconsistent, seasonal variation in fat content. The minimum fat percentages in "quarters" was found in October, 1918, and January and February, 1919. Yet the period of maximum fat content extended from July, 1919 to September, 1919. Unfortunately, sardines of these two groups were not available later in the year and so it is impossible to say whether there is always to be expected a sudden decrease in the fat content of small sardines in the fall. It does seem certain that their maximum fat content is reached during the late summer.

It has been shown in a previous paper that mackerel were fatter in the fall of 1919 than in the previous fall. It was also true, as indicated in Tables I and IV, that large sardines were fatter in the season of 1919-20 than in the previous season. This is true month for month in the case of "small ovals" and with the apparent exception of January, 1919, is true of "large ovals" as well. This one exception may be explained by the high average weight of the January, 1919, samples, 225 gm. contrasted with 170 gm., the average weight of the January, 1920, samples.

Fairly consistent variations in the fat contents of large sardines are revealed by a study of Tables I and IV. The fat contents were at a maximum from December to February or March and appeared to drop off rapidly in April of both 1919 and 1920. Evidence was found that this sudden dropping off may have a different explanation from the obvious one. Thus in Table V are shown the analyses of sardines of three different dates during the transition period from high to low fat content. The fish analyzed on March 29 and April 12 were relatively fat while those analyzed at an intermediate date, April 8, were thin. This strongly indicates that there is much difference in schools at this season of the year and suggests that the fish of March 29 and April 12 were from the same or similar schools while those of April 8 were from a school of thin fish.

The opinion has been expressed that the decrease in fat content of the large sardine is closely related to the development of the gonads and the approach of the spawning season. The fact that the time of low fat content and the spawning period are nearly coincident lends support to this view. Considerable attention has

TABLE V.

*Variation in the Composition of Sardines from Different Schools.*

Description of fish analyzed.				Composition of the edible portion.			
Date.	Average weight per fish.	Weight of gonads divided by total weight.	Average length.	Solids.	Ether extract.	Total nitrogen.	Protein (N $\times$ 6.25).
1920	gm.		cm.	per cent	per cent	per cent	per cent
Mar. 29.....	80	0.037	19.2	25.56	3.20	3.36	21.00
" 29.....	100	0.036	21.0	25.92	4.20	3.24	20.25
" 29.....	120	0.031	21.8	29.93	9.11	3.17	19.81
" 29.....	140	0.058	22.4	34.03	14.09	2.96	18.50
" 29.....	160	0.052	23.7	34.57	14.78	2.93	18.31
" 29.....	180	0.054	24.0	35.77	15.89	2.93	18.31
" 29.....	200	0.044	25.1	35.27	15.06	2.98	18.63
" 29.....	220	0.048	25.9	35.25	15.11	2.94	18.37
" 29.....	240	0.048	26.9	33.43	13.39	2.99	18.69
Apr. 8.....	100	0.028	21.1	23.30	2.13	3.16	19.75
" 8.....	120	0.025	22.0	23.63	3.23	3.08	19.25
" 8.....	140	0.033	23.1	24.04	3.38	3.11	19.44
" 8.....	160	0.043	24.0	23.55	2.65	3.14	19.63
" 8.....	180	0.027	25.4	21.66	0.88	3.11	19.44
" 8.....	200	0.038	26.2	23.31	3.42	3.00	18.75
" 8.....	220	0.042	27.0	27.09	6.25	3.10	19.37
" 12.....	100	0.049	20.5	29.19	7.89	3.15	19.69
" 12.....	160	0.082	23.7	31.84	10.95	3.09	19.31
Average of 100 and 160 gm. samples from each group.							
Mar. 29.....	130	0.044	22.3	30.24	9.49	3.08	19.25
Apr. 8.....	130	0.035	22.5	23.42	2.39	3.15	19.69
" 12.....	130	0.065	22.1	30.51	9.42	3.12	19.50

been devoted to this question of the relation of percentage of fat to degree of sexual development. In Table VI are shown the analyses of ovaries and testes of the sardine in March, April, and June, 1920. It is evident that there is no great change in the composition of the reproductive organs as spawning season



approaches. The only consistent change is a decreasing fat percentage both in the ovaries and testes.

Table VII shows the changing weight of the gonads as the season advances. The maximum was reached during April or May at which time spent fish began to appear; at this point the relative

TABLE VI.  
*Composition of the Gonads of Sardines.*

No.	Sample.	Date.	Solids.	Ether extract	P <sub>2</sub> O <sub>5</sub>	Total nitrogen.	Protein (N×6.25).
		1920	per cent	per cent	per cent	per cent	per cent
1	Testes.	Mar. 25	20.84	2.59	1.28	2.80	17.50
2	Ovaries.	" 25	27.66	4.54	1.08	3.24	20.25
3	Testes.	Apr. 12	20.77	2.39	1.21	2.91	18.19
4	Ovaries.	" 12	29.59	3.76	1.17	3.66	22.88
5	Testes.	June 3	20.52	1.07	1.25	2.97	18.56
6	Ovaries.	" 3	23.89	2.12	0.98	3.00	18.75

TABLE VII.  
*Ratio of Weight of Gonads to Total Body Weight.*

No.	Date.	Ratio for "small ovals."	Ratio for "large ovals."
	1920		
1	Jan. 20	0.015	0.018
2	Feb. 2	0.017	0.023
3	" 17	0.022	0.029
4	Mar. 3	0.025	0.028
5	" 10	0.036	0.035
6	" 19	0.047	0.045
7	" 29	0.035	0.051
8	Apr. 8	0.029	0.037
9	" 12	0.049	0.082
10	" 19	0.047	0.067
11	" 27	0.026	0.057
12	May 5	0.046	0.072
13	" 17	0.023	0.046
14	June 3	0.009	0.052

weight of gonads began to decrease as the proportion of spent fish increased. It was found that "small ovals" tend to spawn before the larger sardines. This tendency is shown by the data of June 3 in Table VII. Gonads composed only 0.009 of the total weight of "small ovals" and 0.052 of the weight of "large

ovals." Of the fish analyzed on June 3, 28 out of 30 "small ovals" were spent while only 12 out of 40 "large ovals" were spent. The maximum weight of gonads found was on April 12 (Table VII, No. 9) and was 0.082 of the body weight. The analysis of this particular sample is shown in Table V (160 gm. sample of April 12)—the fat content was relatively high, 10.95 per cent, showing that a considerable growth of the reproductive organs can take place without drawing to any great extent on the reserve store of fat.

TABLE VIII.

*Composition of Sardines of Different Degrees of Sexual Development.*

Description of fish analyzed.				Composition of the edible portion.			
Date.	Average weight per fish.	Weight of gonads divided by total weight.	Average length.	Solids.	Ether extract.	Total nitrogen.	Protein (N×6.25).
1920	gm.		cm.	per cent	per cent	per cent	per cent
Feb. 23 (a)..<	154	0.040	23.3	38.89	18.18	2.92	18.25
" 23 (b)..<	151	0.017	23.0	37.86	18.13	2.93	18.31
" 23 (c)..<	149	0.025	23.2	37.42	17.21	2.96	18.50
" 23 (d)..<	151	0.016	23.3	36.73	16.27	3.03	18.94
Apr. 2 (a)..<	130	0.082		34.22	14.35	2.88	18.00
" 2 (b)..<	124	0.052		32.73	12.58	2.92	18.25
" 2 (c)..<	136	0.044		33.70	14.07	2.92	18.25
" 2 (d)..<	131	0.029		31.52	11.46	2.90	18.13

(a) represents the values found with relatively mature males; (b), relatively immature males; (c), relatively mature females; and (d), relatively immature females.

This same question was approached from another angle. Advantage was taken of the fact that the reproductive organs are of different degrees of maturity in sardines of the same size from the same school. On two occasions fifty or more fish of approximately the same weight were selected and from these ten relatively mature and ten relatively immature fish of each sex were segregated and analyzed. The results, shown in Table VIII, are remarkable for their close similarity. The only consistent variation is that the samples of highest fat content are from the more mature fish. This strongly indicates that in sardines the develop-

ment of the reproductive organs is not closely related to the decreasing fat content although both take place at about the same time.

In some cases the relation between the fat content of fish and the sea temperature has been established. It has been shown, for example, that sea temperature is a factor in the variable fat content of the herring of European waters. It has been found that the minimum sea temperature at the surface off southern California is reached in January or February while the maximum is reached in July or August with a range of 6 or 7°C.<sup>1</sup> Since the fat content of large sardines is near the maximum in January or February, the possibility of more than a remote relationship between these factors in the case of the sardine must be slight.

Aside from the determination of constituents shown in the tables, several determinations of glycogen in sardines have been made. Three of these five determinations yielded 0.50, 0.17, and 0.22 per cent of glycogen in the edible portion. The other two showed no trace of glycogen but, as these two samples were from fish which had been out of the water for several hours while the other three were based on live fish, the absence of glycogen may have been due to hydrolysis. On one occasion several hundred grams of flesh from live sardines were rapidly ground and mixed with a strong potassium hydroxide solution. Instead of hydrolysis of the glycogen and determination of the sugar in the usual way, the glycogen was separated and purified by repeated reprecipitation with alcohol. In this way several decigrams of an amorphous brown powder were obtained which gave an opalescent aqueous solution which produced a red color with iodine.

#### CONCLUSIONS.

Considerable variation in the composition of individual sardines of the same size and from the same school may occur.

Small sardines were found to have a maximum fat content in the summer months.

With some exceptions, other factors being the same, the fat content of sardines increases with the increasing size of the fish.

<sup>1</sup> McEwen, G. F., Summary and interpretation of the hydrographic observations, made by the Scripps Institution for Biological Research of the University of California, 1908 to 1915, Berkeley, 1916.

Marked variations in the fat content of a seasonal nature were found in large sardines, the percentage of fat dropping from a maximum in December or earlier to a minimum in May. This variation was more-extreme in 1918-19 than in the following season.

Great difference in the fat content of sardines of the same size from different schools was observed. The migration of schools may be related to the sudden decrease in fat content that takes place in April of each season.

No evidence that the growth of the reproductive organs draws to any great extent on the reserve store of fat was derived.

The relation between the percentage of fat in the sardine and the sea temperature, if any, is remote.

There are appreciable percentages of glycogen in the flesh of the sardine.



# THE ESTIMATION OF CREATININE IN THE PRESENCE OF ACETONE AND DIACETIC ACID.

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Since the adaptation by Folin of the Jaffé reaction to the quantitative estimation of creatinine in urine, the procedure has been made the basis of much important research bearing on the problem of the metabolism of creatine and creatinine. The accuracy and reliability of the method have only been questioned in the case of pathological urines, more particularly in specimens containing acetone and diacetic acid. Many of the experiments dealing with this question that were described in the literature offer inconclusive and contradictory evidence as to the exact mode and extent of interferences of the acetone bodies with the determination of creatinine by the Folin method. Klercker<sup>1</sup> stated that large amounts of acetone cause a rapid fading of the creatinine color, while van Hoogenhuyze and Verploegh<sup>2</sup> claimed that the color is at first too dark, but soon fades to a point where it gives correct creatinine reading. Similar results were reported by Rose<sup>3</sup> with regard to the effect of diacetic acid on the color of creatinine and alkaline picrate. His attention was arrested by the assertion of Krause<sup>4</sup> that diacetic acid causes a measurable increase in color, leading ultimately to too low creatine values. From his own investigation, Rose draws the conclusion that diacetic acid, if present in amounts not exceeding 0.25 per cent,

<sup>1</sup> cf Klercker, K. O., *Biochem. Z.*, 1907, iii, 45.

<sup>2</sup> van Hoogenhuyze, C. J. C., and Verploegh, H., *Z. physiol. Chem.*, 1908, lvi, 161.

<sup>3</sup> Rose, W. C., *J. Biol. Chem.*, 1912, xii, 73.

<sup>4</sup> Krause, R. A., *Quart. J. Exp. Physiol.*, 1910, iii, 289.

gives an increase in color, which, however, soon fades and offers therefore no serious obstacle to the correct estimation of creatinine. In concentrations larger than 0.25 per cent, the color due to diacetic acid is more persistent and hence objectionable. He furthermore states that acetone in all concentrations is without influence on the reading.

The conclusions with regard to the effect of diacetic acid are far from incontestable, for the reason that Rose used the ethyl ester of diacetic acid and not the acid itself in his experiments. As was shown by Graham and Poulton,<sup>5</sup> and we can confirm their findings, these two substances do not behave in an analogous manner with respect to their effect on the color reaction of creatinine. The same criticism may be applied to the work of Wolf and Osterberg,<sup>6</sup> who found that 1.0 per cent of ethyl acetoacetate caused no marked change in the creatinine reading.

In contrast to the results of Rose stand the reported findings of Greenwald<sup>7</sup> that acetone in amounts greater than 0.5 per cent gives at first an undue increase in color, which soon drops below its normal value on account of fading. In the presence of diacetic acid the creatinine color is always too light. Greenwald estimated the amount of diacetic acid added to urine by the intensity of the ferric chloride reaction and made no attempt to determine the minimum amount of diacetic acid which may possibly interfere. Graham and Poulton<sup>5</sup> detail careful experiments in which they have added graded quantities of acetone, ethyl acetoacetate, and sodium acetoacetate to urine and studied the effect of these substances on the creatinine color. They concluded that acetone, if less than 0.2 per cent, causes no error; with larger amounts the color is lighter than normal. Ethyl acetoacetate in small amounts (0.1 to 0.7 per cent) gives a lighter color; larger amounts give a darker color which increases on standing. Sodium acetoacetate, even in small amounts, causes a lighter color; with larger amounts the color is still lighter and fades rapidly on standing. Consequent upon these findings, they have investigated the question of alleged creatinuria in carbohydrate starvation. As a result of

<sup>5</sup> Graham, G., and Poulton, E. P., *Proc. Roy. Soc. London, Series B*, 1913-14, lxxxvii, 205.

<sup>6</sup> Wolf, C. G. L., and Osterberg, E., *Am. J. Physiol.*, 1911, xxviii, 71.

<sup>7</sup> Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 87.

their study, they have come to the conclusion that the figures for urine creatine in carbohydrate starvation reported by previous observers merely represented discrepancies between correct and incorrect creatinine determinations, due to the respective absence and presence of diacetic acid in the urine after and before heating of the specimen for the conversion of creatine to creatinine.

In view of these conflicting statements with regard to the effect of acetone and diacetic acid on the estimation of creatinine and their bearing on the problem of creatine metabolism, as pointed out by Graham and Poulton, it was deemed worth while to subject the matter to a critical experimental examination. We have studied the effect of added acetone, diacetic acid, and ethyl acetoacetate on the color reaction of creatinine in pure solution and in urine.

*Effect of Acetone on the Color Reaction of Creatinine in Pure Solution.*—A stock solution of pure creatinine was made by dissolving 1.0 gm. in 1,000 cc. of 0.1 N HCl (1.0 cc. – 1.0 mg. of creatinine), 2 and 5 cc. portions, respectively, were placed in a 500 cc. volumetric flask, acetone and water were added to a volume of 10 cc. The solution was then treated with 15 cc. of saturated picric acid and 5 cc. of 10 per cent sodium hydroxide solution, allowed to stand for 8 to 10 minutes, and after diluting to the mark, compared with a standard creatinine solution similarly treated with picric acid and alkali. The results are given in Table I.

*Effect of Added Acetone on the Determination of Creatinine in Urine.*—A quantity of urine was boiled in an open flask for about 10 minutes to remove all traces of acetone and diacetic acid. It was then cooled to room temperature and its creatinine content estimated, using a 0.5 N  $K_2Cr_2O_7$  solution as a standard. Different portions of the sample were mixed with varying amounts of acetone and each analyzed for creatinine (Table II).

Tables III and IV give the results of experiments with ethyl acetoacetate.

*Experiments with Diacetic Acid and Acetone.*—A pure solution of diacetic acid was prepared by the hydrolysis of its ethyl ester according to the directions of Ceresole.<sup>8</sup> Just before using, the solution was placed in a tall cylinder and a current of acetone-free

<sup>8</sup> Ceresole, M., *Ber. chem. Ges.*, 1882, xv, 1871.



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air slowly drawn through it for about 45 minutes to remove traces of acetone that have been formed from the spontaneous decomposition of the acid. A measured volume of it was then transferred to a Kjeldahl flask, diluted with several volumes of water, acidified with  $\text{H}_2\text{SO}_4$ , and distilled into water. The acetone caught in the

TABLE I.

*Effect of Acetone on the Color Reaction of Creatinine in Alkaline Picrate.*

Creatinine taken.	Acetone added to 10 cc		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
2.0	0.0	0.0	10.0	2.0	0.0
2.0	20.0	0.20	10.0	2.0	0.0
2.0	40.0	0.40	10.1	1.98	- 1.0
2.0	50.0	0.50	10.1	1.98	- 1.0
2.0	60.0	0.60	10.3	1.94	- 3.0
2.0	70.0	0.70	10.6	1.88	- 6.0
2.0	80.0	0.80	10.8	1.85	- 7.5
2.0	100.0	1.00	11.5	1.74	-13.0
2.0	150.0	1.50	12.8	1.55	-22.0
2.0*	200.0	2.0	15.7	1.27	36.5
5.0	0.0	0.0	10.0	5.0	0.0
5.0	40.0	0.40	10.0	5.0	0.0
5.0	50.0	0.50	10.0	5.0	0.0
5.0	60.0	0.60	10.1	4.95	- 1.0
5.0	70.0	0.70	10.3	4.85	- 3.0
5.0	90.0	0.90	10.5	4.76	- 4.8
5.0	120.0	1.20	11.0	4.54	- 9.2
5.0	180.0	1.8	11.7	4.27	-14.6
5.0	500.0	5.0	14.0	3.57	-28.6
5.0†	800.0	8.0	33.0	1.51	-65.8

\* Standard, 2.0 mg. of pure creatinine set at 10 mm.

† Standard, 5.0 mg. of pure creatinine set at 10 mm.

receiver was then determined by the Messinger titration. From the figures obtained, the percentage of diacetic acid in the solution was calculated. Definite quantities of this solution were added to known amounts of creatinine in pure solution, and in urine, and their effect noted. The results are summarized in Tables V and VI.

## DISCUSSION.

The data in the tables show consistently that, barring a few minor exceptions, our results are in substantial agreement with those obtained by Graham and Poulton. Acetone in large amounts undoubtedly fades the creatinine color from the very outset. We are inclined to set the upper limit of allowable acetone concentration at 0.50 per cent. The figures presented by Graham and

TABLE II.

*Effect of Acetone on the Determination of Creatinine in Urine.\**

Acetone added to 10 cc. of urine.		Color.	Creatinine in 100 cc of urine.	Error.
<i>mg.</i>	<i>per cent</i>	<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
0.0	0.0	7.7	105.19	0.0
40.0	0.4	7.7	105.19	0.0
50.0	0.5	7.7	105.19	0.0
60.0	0.6	7.8	103.87	- 1.19
70.0	0.7	7.9	102.53	- 2.52
90.0	0.9	8.4	96.42	- 8.35
120.0	1.2	8.8	92.04	-12.46
150.0	1.5	9.1	89.01	-15.38
200.0	2.0	9.8	82.65	-21.42
0.0	0.0	8.1	100.00	0.0
30.0	0.3	8.1	100.00	0.0
50.0	0.5	8.2	98.78	- 1.22
60.0	0.6	8.1	100.00	0.0
70.0	0.7	8.4	97.62	- 2.38
80.0	0.8	8.6	94.18	- 5.82
90.0	0.9	8.7	93.0	- 7.00
100.0	1.0	9.0	90.0	-10.00
150.0	1.5	13.5	60.0	-40.00
300.0	3.0	16.0	50.62	-49.38

\* Standard, 0.5 N potassium dichromate. All tests, giving higher reading, fade on standing.

Poulton show that 0.17 per cent acetone had no effect on the reading, but 1.0 per cent gave a markedly lighter color. The gap between 0.17 and 1.0 per cent is obviously a wide one. We can say that the fading effect of acetone does not begin markedly to show itself until a concentration of 0.70 per cent has been reached. With no amount of acetone did we observe an increase in color (Tables I and II).

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TABLE III.

*Effect of Ethyl Acetoacetate on Color of Pure Creatinine.\**

Creatinine taken.	Ester added to 10 cc.		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
5.0	0.0	0.0	10.0	5.0	0.00
5.0	5.0	0.05	10.0	5.0	0.00
5.0	10.0	0.10	10.1	4.95	- 1.00
5.0	20.0	0.20	10.3	4.85	- 3.00
5.0	50.0	0.50	9.8	5.10	+ 2.00†
5.0	60.0	0.60	9.0	5.55	+11.00†
5.0	100.0	1.00	8.0	6.25	+25.00†

\* Standard, 5 mg. of pure creatinine set at 10 mm.

† Did not fade after standing for 10 minutes.

TABLE IV.

*Effect of Ethyl Acetoacetate on Determination of Creatinine in Urine.\**

Ester added to 10 cc.		Color.	Creatinine in 100 cc. of urine.	Error.
mg.	per cent	mm.	mg.	per cent
0.0	0.0	12.2	66.39	0.00
10.0	0.10	12.5	64.80	- 2.39
20.0	0.20	12.6	64.28	- 3.17
50.0	0.50	12.4	65.32	- 1.61
60.0	0.6	12.0	67.5†	+ 1.67†
100.0	1.0	11.0	73.63†	+10.90†
200.0	2.0	9.0	90.0†	+35.56†

\* Standard, 0.5 N potassium dichromate.

† No fading, but slight increase of color after 10 minutes.

TABLE V.

*Effect of Diacetic Acid on Color of Pure Creatinine in Alkaline Picrate.\**

Creatinine taken.	Diacetic acid : 10 cc.		Color.	Creatinine found.	Error.
mg.	mg.	per cent	mm.	mg.	per cent
5.0	0.00	0.00	10.0	5.00	0.00
5.0	1.5	0.015	10.0†	5.00	0.00
5.0	2.1	0.021	10.3	4.85	- 3.00
5.0	3.0	0.030	10.7	4.67	- 6.60
5.0	4.5	0.045	11.2	4.46	-10.80
5.0	9.0	0.09	12.3	4.06	-18.80
5.0	15.0	0.15	13.5	3.70	-26.00

\* Standard, 5 mg. of pure creatinine treated the same as the unknown.

† Fades after about 5 minutes to 10.2. All other tests fade even more rapidly.

Ethyl acetoacetate in small amounts causes a fading in the creatinine color, while in larger amounts it causes an increase in color. The line of demarkation is difficult to establish, as the two

TABLE VI.

*Effect of Diacetic Acid on the Determination of Creatinine in Urine.*

Acid added to 10 cc.		Color.	Creatinine in 100 cc.	Error.
<i>mg.</i>	<i>per cent</i>	<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
0.0	0.0	3.0	270.00	0.00
2.1	0.021	3.1	261.29	- 3.22
3.0	0.030	3.2	253.12	- 6.25
4.5	0.045	3.4	238.23	-12.13
6.0	0.06	3.8	213.16	-21.05
9.0	0.09	4.1	197.56	-26.82
15.0	0.150	5.5	147.27	-45.45
0.0	0.0	5.1	158.82	0.00
2.1	0.021	5.3	152.83	- 3.51
3.0	0.030	5.5	147.27	- 7.27
4.5	0.045	5.7	142.10	-10.52
6.0	0.060	6.1	132.78	-16.39
9.0	0.090	6.9	117.39	-26.08
15.0	0.150	8.2	98.78	-37.80
0.0	0.0	6.8	119.11	0.00
1.107	0.011	7.0	115.71	- 2.85
2.214	0.022	7.3	110.16	- 7.51
3.321	0.033	7.7	105.19	-11.68
5.535	0.55	8.4	96.42	-19.05
0.0	0.0	8.3	98.79	0.00
2.77	0.027	9.8	82.55	-16.42
5.535	0.055	10.8	75.00	-24.08
11.07	0.110	13.0	62.30	-36.93
0.0	0.0	12.5	64.80	0.00
1.107	0.011	13.0	62.30	- 3.83
2.214	0.022	13.3	60.90	- 6.01
4.428	0.044	15.0	54.00	-16.66
5.535	0.055	16.7	48.50	-25.15

limits seem to merge into each other by an almost insensible gradation (Tables III and IV). This is, of course, of no great importance, since diacetic acid does not occur in urine in the form of its ethyl ester.

Our experiments with diacetic acid bear out the conclusion of Graham and Poulton that this substance, even in small amounts, has a great influence on the creatinine reading. 0.015 or 0.02 per cent causes a perceptible fading, and the color becomes progressively lighter, as the concentration of the acid or the time for which the test is allowed to stand is increased (Tables V and VI).

The tables show uniformly that the influence of a given amount of acetone or diacetic acid on the determination of creatinine is more marked in dilute solutions than in more concentrated ones, and also that the effect is the same, whether the creatinine is in a pure solution or in urine.

The above findings point clearly to the necessity of removing diacetic acid from urine, preparatory to the determination of creatinine in the sample. Of the efforts that have been made towards the development of a suitable technique to serve this purpose, worthy of note are those of Greenwald and of Graham and Poulton.<sup>5</sup> Following the suggestion of Rona, Greenwald has attempted to drive off diacetic acid from urine by boiling, but, after a few trials with urines from cases of muscular dystrophy, abandoned the method because it invariably resulted in higher creatinine values. Whether this was due to the expulsion of diacetic acid, and was therefore the desired result, to the conversion of creatine to creatinine or to the undue concentration of the urine leading to the formation of pigments and chromogenic condensation products; whether any or all of these factors were operative in the production of a greater intensity of color, Greenwald did not ascertain. He adopted the procedure of extracting the urines with ether in a continuous extractor for 2 hours, blowing off the ether, diluting the cool solution to twice its original volume, and developing the creatinine color by the addition of 30 cc. of picric acid and 10 cc. of NaOH. Needless to say that this method is impractical where a great many determinations have to be made. It is tedious and time consuming, and is further limited in its usefulness by the fact that it requires expensive apparatus. The same objections apply, though perhaps with not such great force, to the method described by Graham and Poulton. They direct adding 1.0 cc. of a 10 per cent solution of  $H_3PO_4$  to a measured volume of urine and removing the diacetic acid by distillation at 70°C. and 210 mm. for 45 minutes. Then they neutralize with

NaOH, dilute to a definite volume, and determine the creatinine in an aliquot. Besides being complicated, the method yields satisfactory results only when experimental conditions, as prescribed by Graham and Poulton, are strictly adhered to. Otherwise, there is either an incomplete removal of diacetic acid, or a conversion of creatine to creatinine. At best the danger of error from this latter source is great, because of the employment of a strong acid. Results are also apt to be irregular with urines containing sugar. The method thus makes unusual demands on the time and judgment of the analyst.

In order to circumvent these difficulties we have set about to find a new method for removing diacetic acid from urine. We have tried first to boil urine with water. Unlike Greenwald, we have found that most urines may be thus boiled without causing an appreciable increase in the creatinine figure. Our procedure was as follows:

10 cc. of urine were measured into a 300 cc. flask or beaker, 3 to 4 volumes of water and 5 to 10 mg. of creatine were added, and the liquid was boiled down to its original volume in 5 to 8 minutes. The solution was then cooled in running water, mixed with 15 cc. of saturated picric acid and 5 cc. of 10 per cent NaOH. After 10 minutes the colored solution was transferred quantitatively to a 500 cc. volumetric flask, diluted to the mark, and read in the colorimeter. We have applied the method to a number of urines from a variety of hospital cases. As may be seen from Table VII, a markedly increased creatinine color in boiled urine is an exception rather than the rule.

This increase in color is presumably due to the conversion of creatine to creatinine. That this is not the sole factor is apparent from the fact that when urines containing no creatine at all are boiled, a slightly darker color may result. Again, many specimens containing added creatine may be boiled without any effect on the color. That the conversion of creatine may be insignificant as compared with positive errors from other sources may be shown by the following experiment:

At a given temperature, the rate of conversion of a definite amount of creatine is conditioned by the hydrogen ion concentration of the medium. Now, when the pH is decreased by the addition of sodium acetate, it is reasonable to suppose that the rate of

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TABLE VII.

*Effect of Boiling Urine on the Determination of Creatinine.*

No.	Creatine added to 10 cc.	Unboiled urine.		Boiled urine.		Error.	Remarks.
		Color	Creati- nine.	Color	Creati- nine.		
	mg.	mm.	mg.	mm.	mg.	per cent	
1	0.0	8.5	95.29	8.5	95.29	0.0	Normal.
2	0.0	9.7	83.50	9.8	82.65	-1.01	"
3	0.0	5.0	162.00	5.0	162.00	0.0	"
4	0.0	8.7	93.10	8.5	95.29	0.0	"
5	0.0	7.6	106.57	7.5	108.00	+1.34	Exophthalmic; goit- er; creatine, 233 mg.
6	0.0	4.5	180.00	4.5	180.00	0.0	Diabetes; no sugar or diacetic acid.
7	0.0	5.7	142.10	5.8	139.62	-1.74	Active pulmonary tuberculosis; crea- tine, 119 mg.
8	0.0	7.0	115.71	6.8	119.12	+2.94	Acute nephritis.
9	0.0	8.0	101.25	7.8	103.84	+2.55	Hyperthyroidism; creatine, 110 mg.
10	0.0	10.7	75.70	10.4	77.88	+2.86	Chronic nephritis.
11	0.0	12.4	65.35	12.3	65.85	+0.76	Normal.
12	0.0	6.4	126.56	6.7	120.89	-4.48	Normal; initial reac- tion, alkaline.
13	10.0	10.2	79.41	9.8	82.65	+4.08	Normal.
14	10.0	4.5	180.00	4.5	180.00	0.0	"
15	10.0	6.9	117.29	6.9	117.29	0.0	"
16	10.0	6.4	126.56	6.4	126.56	0.0	"
17	10.0	7.9	102.54	7.4	109.46	+6.75	"
18	10.0	9.2	88.04	9.0	90.00	+2.23	Lues.
19	10.0	9.0	90.00	9.0	90.00	0.0	Pneumonia.
20	10.0	12.6	64.28	12.2	66.39	+3.28	Diabetes.
21	10.0	10.5	77.14	10.4	77.88	+0.95	Auricular fibrilla- tion.
22	10.0	13.2	61.37	12.6	64.28	+4.57	Pneumonia.
23	10.0	7.0	115.71	6.7	120.89	+4.47	"
24	10.0	12.6	64.28	11.2	72.32	+12.50	Cerebrospinal syph- ilis.
25	10.0	5.5	147.27	5.5	147.27	0.0	Pernicious anemia.
26	10.0	6.7	120.89	6.7	120.89	0.0	" "
27	10.0	8	101.25	7.3	110.95	9.52	Bronchitis.
28	10.0	5.6	144.64	5.6	144.64	0.0	Tuberculosis.

Creatinine values are for 100 cc. of urine.

conversion of creatine will be retarded, yet the reading of a urine that yields a higher creatinine value on boiling will be the same whether the sample has been boiled for the same length of time with or without the buffer. It is evident then that some other element besides the change of creatine into creatinine enters into the production of a greater intensity of color. The fact that normal urines (diacetic acid and creatine-free) to which glucose has been added yield a considerable increase of color after boiling (Tables VIII and IX) would seem to indicate that condensation products play an important rôle in the process. In working with dog urines the fact has also developed that during boiling the reaction often becomes less acid or even slightly alkaline, in consequence of which there is a slight loss of creatinine.

Because of these occasional irregularities of the creatinine readings, encountered when urines are boiled with water, we do not feel justified in recommending the procedure for general adoption. A much safer method consists in boiling the urine after the addition of a substance which lowers the boiling point and at the same time has no effect on either creatine or creatinine. Methyl alcohol has been found to answer these requirements. It is also relatively inexpensive and readily obtainable.<sup>9</sup>

To show that methyl alcohol does not change the composition or properties of creatine and creatinine, a mixture of an aqueous solution of these two compounds was slowly boiled over an asbestos mat with about 5 volumes of the reagent until the temperature reached 100°C. The solution was then cooled, picric acid and alkali were added, and the color was developed in the usual way. The correct reading for the creatinine content of the solution was obtained. The same result was obtained when the solution was slightly acidified with lactic or acetic acid and treated as just described. Pure solutions, as well as urines to which excessive quantities of diacetic acid were added, were found, after this treatment, to be negative to the ferric chloride reaction. The legal reaction occasionally revealed the presence of traces of acetone, but these had no measurable influence on the creatinine reading. Urines containing added glucose (Tables VIII and IX) were

<sup>9</sup> The use of methyl alcohol in this connection was suggested by Dr. S. R. Benedict, to whom the writer is indebted for much helpful advice during the course of this work.



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TABLE VIII.

*Experiments with Methyl Alcohol Boiling to Remove Diacetic Acid from Urine.*

No.	Unboiled.		Acid added.		Creatine added.	Boiled + alcohol.		Error.
	Color.	Creatinine.				Color.	Creatinine.	
	mm.	mg.	mg.	per cent	mg.	mm.	mg.	per cent
1	11.7	69.23	11.07	0.1107	10.0	11.7	69.23	0.0
2	7.2	112.50	11.07	0.1107	10.0	7.2	112.50	0.0
3	7.7	105.19	11.07	0.1107	10.0	7.7	105.19	0.0
4	7.2	112.50	11.07	0.1107	10.0	7.2	112.50	0.0
5	5.2	155.76	11.07	0.1107	10.0	5.2	155.76	0.0
6	10.3	78.64	11.07	0.1107	10.0	10.3	78.64	0.0
7	8.0	101.25	11.07	0.1107	10.0	8.0	101.25	0.0
8	6.8	119.11	11.07	0.1107	10.0	6.8	119.11	0.0
9	8.0	101.25	11.07	0.1107	10.0	8.0	101.25	0.0
10	5.7	142.10	11.07	0.1107	10.0	5.8	139.62	-1.74
11	10.4	77.80	11.07	0.1107	10.0	10.4	77.80	0.0
12	12.8	63.51	11.07	0.1107	10.0	12.8	63.51	0.0
13	8.7	93.1	11.07	0.1107	10.0	8.7	93.1	0.0
14	6.4	126.56	11.07	0.1107	10.0	6.4	126.56	0.0
15	12.8	63.51	11.07	0.1107	10.0	12.8	63.51	0.0
16	15.0	54.00	11.07	0.1107	10.0	15.0	54.00	0.0
17	12.5	64.80	11.07	0.1107	10.0	12.5	64.80	0.0
18	5.8	139.62	11.07	0.1107	10.0	5.9	137.28	-1.60
19	9.4	86.16	11.07	0.1107	10.0	9.4	86.16	0.0
20	5.9	137.28	11.07	0.1107	10.0	5.8	139.62	+1.70
21	8.3	97.59	0.0	0.0	0.0	8.3	97.59	0.0
21	8.3	97.59	2.77	0.0277	2.0	8.3	97.59	0.0
21	8.3	97.59	5.53	0.0553	2.0	8.3	97.59	0.0
22	7.5	108.00	0.0	0.0	2.0	7.5	108.00	0.0
23	12.5	64.80	1.107	0.0117	2.0	12.5	64.80	0.0
23	12.5	64.80	2.214	0.02214	2.0	12.5	64.80	0.0
23	12.5	64.80	4.43	0.0443	2.0	12.5	64.80	0.0
23	12.5	64.80	5.53	0.0553	2.0	12.5	64.80	0.0
24	7.7	105.19	70.40	0.704	8.0	7.7	105.19	0.0
25	8.3	97.59	70.40	0.704	8.0	8.3	97.59	0.0
26	8.0	101.25	70.40	0.704	8.0	8.0	101.25	0.0
27	9.0	90.0	70.40	0.704	8.0	9.0	90.0	0.0

Creatinine values are for 100 cc. of urine.

but very slightly darker than normal after having been boiled with methyl alcohol, and gave correct creatinine readings; while the same urine boiled with water gave a darker color with a brownish tint, which made colorimetric comparison somewhat difficult.

TABLE IX.

*Experiments with Dog Urines.*

No.	Unboiled.		Boiled + alcohol.		Error.	Boiled + water.		Error.	Graham and Poulton distillation.		Error.
	Read- ing.	Creati- nine.	Read- ing.	Creati- nine.		Read- ing.	Creati- nine.		Read- ing.	Creati- nine.	
	mm.	mg.	mm.	mg.	per cent	mm.	mg.	per cent	mm.	mg.	per cent
28	8.7	93.10	8.7	93.10	0.0						
29	12.6	64.28	12.6	64.28	0.0				10.5	77.14	+20.00
30	8.9	91.01	8.9	91.01	0.0	9.1	89.01	- 2.19	12.0	67.5	-24.72
31	9.1	89.01	9.1	89.01	0.0	8.6	93.07	+ 4.56	9.1	89.01	0.0
32	12.2	66.39	12.2	66.39	0.0	12.0	67.50	+ 1.67	11.0	73.63	+10.90
33	11.8	68.64	11.8	68.64	0.0	12.2	66.48	- 1.68	11.8	68.64	0.0
34	9.0	90.00	9.1	89.01	-1.1	8.9	91.01	+ 1.12	8.7	93.10	+ 7.66
35	9.9	81.81	9.9	81.81	0.0	10.0	81.00	- 0.99	9.6	84.37	+ 3.12
36	10.2*	79.41	10.2	79.41	0.0	8.0	101.25	+21.52	8.3	97.59	+22.89
37	8.0†	101.25	8.0	101.25	0.0	7.5	108.00	+ 6.66	8.7	93.10	-14.71
38	8.8†	92.45	8.8	92.45	0.0	8.6	93.07	+ 0.67	7.6	106.57	+15.27

\* 10.0 per cent of glucose added.

† 5.0 " " " " " "

Diacetic acid added to each sample = 70.40 mg. to 10.0 cc. = 0.704 per cent.

Creatine added to each sample = 5.0 mg. to 10.0 cc.

Values are mg. per 100.0 cc. of urine.

Standard = 0.5 N dichromate.

The details of the proposed method are as follows: 10 cc. of urine are introduced into a 300 cc. Erlenmeyer or round bottom flask containing a few glass beads. If the urine is alkaline the reaction should be adjusted with strong HCl to be that due to weak organic acids; that is, it should be red to litmus but not blue to Congo red. 5 volumes of methyl alcohol are added and the mixture is slowly boiled over an asbestos mat until 1 or 2 minutes

after the temperature has risen to 100°C.<sup>10</sup> This should take not less than 15 minutes. The flask is now thoroughly cooled in running water, its contents are mixed with 15 cc. of saturated picric acid and 5 cc. of 10 per cent NaOH, at the end of 8 minutes washed quantitatively into a 500 cc. flask, diluted to mark, and read.

The time allowed for decomposing the diacetic acid and driving off the acetone formed is ample enough for such concentrations of the interfering substance as are ordinarily met with in pathological urines. We have repeatedly freed 10 cc. of urine from as much as 70.4 mg. (0.704 per cent) of added diacetic acid by this method. But for this it is necessary to keep the liquid in a state of active boiling during the entire period of 15 minutes. The mixture usually begins to boil around 75°C. and stays at that temperature for about 8 minutes. After that the temperature gradually rises to 100°, and the contents of the flask will have returned to their original volume. Boiling a little longer beyond this point does no harm and rather insures the expulsion of traces of acetone. At first we have tried boiling on a water bath. Under these conditions the temperature can be kept constant at about 85°C. for a considerable length of time, but this was found unnecessary. In several instances, when heating in this way was unduly prolonged, an appreciable increase in the creatinine figure was obtained.

One other precaution is necessary; that is to make sure that the urine is thoroughly cooled before adding the picric acid and the sodium hydroxide. Otherwise, an unduly dark color will result.

Table VIII shows some results obtained by the method.

#### SUMMARY.

The influence of acetone, ethyl acetoacetate, and diacetic acid on the color reaction of creatinine in alkaline picrate was studied. It was found that these substances interfere with the colorimetric determination of creatinine in pure solution as well as in urine.

A method for removing diacetic acid from urine has been described.

<sup>10</sup> The methyl alcohol was usually distilled off through a Liebig condenser. If the alcohol is boiled off from an open vessel the operation should be carried out in a hood on account of the toxicity of methyl alcohol vapors. The recovered methyl alcohol can be readily purified by refluxing with acid mercuric sulfate, filtering off the precipitate formed, and fractionating the filtrate.

# ON THE STRUCTURE OF THYMUS NUCLEIC ACID AND ON ITS POSSIBLE BEARING ON THE STRUCTURE OF PLANT NUCLEIC ACID.

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(Received for publication, July 1, 1921.)

The polynucleotide structure of both yeast and animal nucleic acids is generally accepted; the question of the mode of linking of individual nucleotides continues to be the subject of discussion and of disagreement between individual workers. Thannhauser, Jones, and the present writer, have each put forth a different theory of the structure of the plant nucleic acid. Each author has criticized the theories of the other two. As a result of these criticisms, Thannhauser<sup>1</sup> has modified his theory and incorporated in his formulation the views of Jones. Thus the criticism of the present author of the theory of Jones applies also to that of Thannhauser. It is peculiar that Jones, in the latest edition of his monograph, in discussing the theories of the structure of yeast nucleic acid does not at all refer to the theory of the present writer.

The point of contention is the following: Are the nucleotides united one to another in an ether linking through their carbohydrates, or in ester form, the phosphoric acid of one combining with the carbohydrates of the other. Jones, and with him Thannhauser, accepts the ether linking, the present writer the ester linking. Since the original evidence on which Jones and Thannhauser have based their theories was proved an experimental error, Jones<sup>2</sup> has furnished two new experiments in support of his theory. The first is the following:

The curve expressing the rate of hydrolysis of yeast nucleic acid is identical with that of a mixture of the four nucleotides. Accepting the experiment as correct, what does it demonstrate? It proves that the union between individual nucleotides is more

<sup>1</sup> Thannhauser, S. J., and Sachs, P., *Z. physiol. Chem.*, 1920-21, cii, 187.

<sup>2</sup> Jones, W., *Am. J. Physiol.*, 1920, lii, 193, 203.

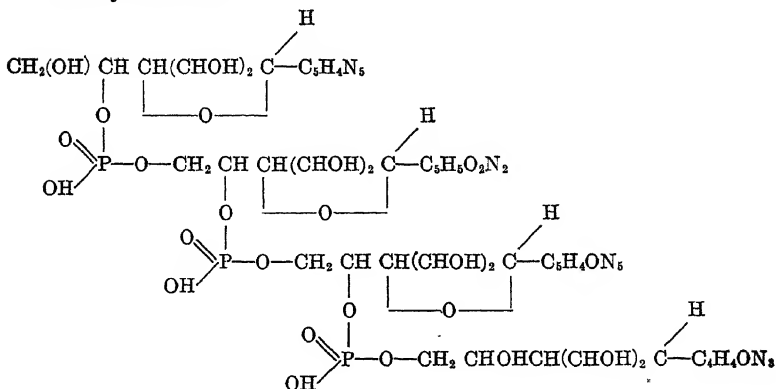
labile than that between the phosphoric acid and the carbohydrate in each nucleotide. It is then self-evident that the first step in the hydrolysis of the nucleic acid molecule is the formation of four nucleotides. The further progress of hydrolysis of the nucleic acid is the same as of four nucleotides. This ready formation of four nucleotides as the initial phase is hard to reconcile with the assumption of Jones of an ether linking between individual nucleotides. On the other hand, this observation is consistent with the theory of the present writer. Truly, the cleavage of nucleic acid into mononucleotides should have been impossible if it were otherwise. The view of Levene is substantiated by the observations of Levene, Meyer, and Yamagawa<sup>3</sup> who found that the rate of hydrolysis of phosphoric acid is practically identical whether 1-2-acetone-3- or 5-phosphoric acid-6-benzoyl glucose, or 1-2-acetone-3- or 5-phosphoric acid glucose are hydrolyzed.

The second experimental proof of Jones' theory is the following: By a pancreas enzyme, yeast nucleic acid was cleaved to its nucleotides. At the starting point of the experiment the hydrogen ion concentration of the reacting mixture was brought to  $\text{pH} = 6.4$ , and at the end of the experiment there was no apparent change of the color of the indicator added to the original solution. Hence the author concludes that no acid radicles could be liberated as the result of the hydrolysis. The reasoning is not correct. According to either theory, nucleic acid is a polyphosphoric acid and when brought to a  $\text{pH} = 6.4$ , it possesses considerable buffer effect. Furthermore, each nucleotide is a comparatively weak acid and when liberated does not affect the hydrogen ion concentration of the buffer very markedly. Since the dissociation constant of the nucleotides has not been measured, it is not possible to express the reaction in quantitative terms. Experimentally, however, we convinced ourselves that when a solution of guanosinphosphoric acid is brought to a  $\text{pH} = 6.4$ , it stands the addition of an equal volume of a solution of free guanosinphosphoric acid of the same concentration before any change of color of the indicator can be noticed. Taking further into consideration the fact that a solution of nucleic acid is not perfectly colorless, that an extract of the pancreas always contains a considerable quantity of phosphates and also is not colorless, one easily realizes that the argument of Jones carries but little weight.

<sup>3</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xlii, 323. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1921, xlviii, 233.

Thus, to the mind of the present writer, there exists neither experimental nor theoretical evidence in favor of the theory of the ether linking between individual nucleotides.

On the other hand, the theory of the present writer brings the structure of the yeast nucleic acid in harmony with that of thymus nucleic acid. Levene and Jacobs<sup>4</sup> have described hexothymidindiphosphoric acid and hexocytidindiphosphoric acid obtained on hydrolysis of thymus nucleic acid. They have also isolated hexothymidinmonophosphoric and hexocytidinmonophosphoric acids, and a substance which seemed to them to be a dinucleotide of hexothymidin and hexocytidin. In view of the experience on yeast nucleic acid, it seemed urgent to reinvestigate the question of the dinucleotide. For this purpose larger quantities of the material were required and hence it was attempted to simplify the method of its preparation. The complicated process employed in the older work was abandoned. From the hydrolyzed material the diphosphonucleotides were removed as barium or calcium salt on heating; from the mother liquor of these it was hoped to isolate the monophosphoric nucleotides and the hypothetical dinucleotide. However, under these conditions only the diphosphoric nucleotides could be isolated. It is possible that the monophosphoric acid nucleotides are secondary products, and that the hypothetical dinucleotide is only a mixture of mononucleotides. Thus for the present, until the existence of the dinucleotide is definitely proved, the structure of the thymus nucleic acid should be expressed in analogy with the yeast nucleic acid, namely as follows:



<sup>4</sup> Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 411.

Incidentally the barium salt of the hexothymidindiphosphoric acid was obtained in crystalline form.

For the final solution of the problem of the structure of thymus nucleic acid larger quantities of the material are needed. Since the manufacture of this nucleic acid in Europe has been discontinued, it will have to be prepared with our laboratory facilities. Because of this, the progress of the work will be delayed, but work is now in progress.

#### EXPERIMENTAL.

Commercial animal nucleic acid (Merck) was hydrolyzed in 100 gm. lots. This amount of the acid was heated on flame with reflux condenser for 2 hours with 1,000 cc. of 2 per cent sulfuric acid. The sulfuric acid and the free phosphoric acid are removed by a slight excess of barium hydroxide solution. The excess of this reagent was then removed quantitatively and the solution concentrated under diminished pressure at room temperature to a volume of 300 cc. The nucleotides were then precipitated with a 25 per cent solution of neutral lead acetate. The precipitate was washed repeatedly with cold water and then suspended in water, decomposed by means of hydrogen sulfide gas. The filtrate from the lead sulfide was again concentrated under diminished pressure at room temperature to a volume of 300 cc. This was again neutralized with barium hydroxide, filtered from the slight trace of barium phosphate, and brought to a boil over a free flame. A flocculent precipitate soon appears, which on prolonged boiling assumes a granular character. The filtrate from this precipitate was then concentrated to small volume and again heated as before; generally a second precipitate formed. The material which formed on boiling (Precipitate I) had the composition of the diphosphonucleotides.

The mother liquor from the diphosphonucleotides was precipitated by alcohol (Precipitate II). This precipitate had the elementary composition of monophosphonucleotides. However, when freed from barium and purified through conversion into lead salt and reconversion with barium salt again, there is formed a barium salt insoluble in boiling water. Thus finally, practically all is converted into the diphosphonucleotides.

There seems to be a discrepancy between the present result and that obtained by Levene and Jacobs. Two alternative explanations may be given to the discrepancy; either the monophosphonucleotides found previously by Levene and Mandel and by Levene and Jacobs are products of further decomposition of the diphosphonucleotides formed in the course of further manipulation, or the monophosphonucleotides are missed in the present procedure.

*Composition of Crude Barium Salts.*

No. of sample.	P	N
358 20/21	8.82	5.36
359 20/21	8.27	5.14
361 20/21	7.74	5.89
366 20/21	7.39	5.62
370 20/21	8.27	5.31
362 20/21	5.66	5.96
474 20/21	5.15	6.11

Samples 362 and 474 were combined, freed from barium, converted into lead salt, and this reconverted into barium salt. The greater part settled out on boiling and had the following composition.

No. of sample.	P	N
408 20/21	7.17	5.50

For further purification and for the separation of individual diphosphonucleotides the older procedure was modified. The barium salts were converted into lead salts and these into brucine salts. The brucine salts were fractionated by recrystallization from 35 per cent alcohol until the more insoluble fraction had the elementary composition of the hexothymidindiphosphoric brucine salt. The combined mother liquors were then concentrated and allowed to stand until a crystalline deposit formed. This was again refractionated. After two refractionations the more soluble brucine salt on conversion into the barium salt analyzed for the hexocytidindiphosphoric acid barium salt.



*Analysis of the Brucine Salts.*

The most insoluble fraction of the brucine salt analyzed as follows:

0.1001 gm. of the substance on combustion gave 0.1999 gm. of  $\text{CO}_2$  and 0.0606 gm. of  $\text{H}_2\text{O}$ .

0.2000 gm. of the substance gave 11.4 cc. of nitrogen gas at  $T = 21^\circ\text{C}$ . and  $P = 751$  mm.

0.3000 gm. of the substance gave on fusion 0.288 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 $\text{C}_{11}\text{H}_{18}\text{N}_2\text{P}_2\text{O}_{13}\text{C}_{92}\text{H}_{104}\text{N}_8\text{O}_{16}$     Calculated.    C 54.29, H 6.64, N 6.15, P 2.73.  
 +  $14\text{H}_2\text{O}$ .                                      Found.            C 54.45, H 6.77, N 6.54, P 2.67.

The most soluble fraction analyzed as follows:

0.1004 gm. of the substance gave on combustion 0.1990 gm. of  $\text{CO}_2$  and 0.0591 gm. of  $\text{H}_2\text{O}$ .

0.2000 gm. of the substance gave 12.0 cc. of nitrogen gas at  $T = 23^\circ\text{C}$ . and  $P = 752$  mm.

0.3000 gm. of the substance gave 0.0287 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 $\text{C}_{10}\text{H}_{17}\text{N}_3\text{P}_2\text{O}_{12}\text{C}_{92}\text{H}_{104}\text{N}_8\text{O}_{16}$     Calculated.    C 54.10, H 6.64, N 6.81, P 2.75.  
 +  $14\text{H}_2\text{O}$ .                                      Found.            C 54.05, H 6.58, N 6.85, P 2.67.

This material was then converted into the barium salt. It must be remarked however, that the elementary compositions of the brucine salts of the above two nucleotides do not differ sufficiently one from another to permit identification of the nucleotide on the basis of the analysis of the brucine salts. Often; samples, which on the basis of the analysis of the brucine salt seemed to be the cytidin nucleotide, proved on conversion into the barium salt to be the thymidin nucleotide.

*Conversion of Brucine Salts into Barium Salts.*

For this purpose the brucine salts were dissolved in 35 per cent alcohol, an excess of ammonia water added, and the product allowed to stand in the refrigerator. The brucine was then removed by filtration, the filtrate was again concentrated and allowed to stand to permit further crystallization of brucine. The operation was repeated as long as brucine crystallized out. From the final solution the nucleotides were precipitated by a 25 per cent solution of neutral lead acetate. The lead salt was washed repeatedly with water, filtered, suspended in water, and

freed from lead by means of hydrogen sulfide. The filtrate from lead acetate is concentrated under diminished pressure at room temperature and then converted into the barium salt.

† The hexothymidin salt was then converted into the crystalline form.

*Preparation of Crystalline Hexothymidindiphosphoric Barium Salt.*

9 gm. of the barium salt obtained from the brucine salt were taken up in 500 cc. of water and shaken for 1 hour. Part remained insoluble. All was allowed to stand over night when a crystalline deposit was found covering the undissolved amorphous material. The mixed deposit was then taken in 1.5 liters of water at 30°C. and shaken for 1 hour in a shaking machine. The insoluble part was removed by filtration and the filtrate concentrated under diminished pressure at room temperature to a volume of 350 cc. After several hours of standing there appeared a crystalline deposit consisting of long needles grouped into star-shaped aggregates.

The composition of the substance was the following:

0.1050 gm. of the substance gave 0.0700 gm. of  $\text{CO}_2$  and 0.0182 gm. of  $\text{H}_2\text{O}$ .

0.1719 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 4.97 cc. of 0.1 N acid.

0.2579 gm. of the substance gave on fusion 0.0830 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{13}\text{N}_3\text{P}_2\text{O}_{12}\text{Ba}_2$ .	Calculated.	C 18.37, H 1.97, N 3.89, P 8.62.
	Found.	C 18.11, H 1.93, N 4.04, P 8.97.

*Barium Salt of Hexocytidindiphosphoric Acid.*

This was prepared from the brucine salt in the same manner as the thymidin salt. As yet it has not been converted into the crystalline form.

It analyzed as follows:

0.1052 gm. of the substance gave on combustion 0.0658 gm. of  $\text{CO}_2$  and 0.0260 gm. of  $\text{H}_2\text{O}$ .

0.1870 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.21 cc. of 0.1 N acid.

0.2805 gm. of the substance gave 0.0946 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{13}\text{N}_3\text{P}_2\text{O}_{12}\text{Ba}_2$ .	Calculated.	C 17.05, H 1.86, N 5.97, P 8.81.
	Found.	C 17.05, H 2.12, N 6.17, P 9.40.



## CREATININE AND CREATINE IN MUSCLE EXTRACTS.

### I. A COMPARISON OF THE PICRIC ACID AND THE TUNGSTIC ACID METHODS OF DEPROTEINIZATION.

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Since the publication by Folin (1) in 1914 of the determination of creatinine and creatine in the filtrates from the deproteinization of blood, milk, and tissues by saturated aqueous picric acid solution plus the addition of solid picric acid, criticisms of the method have been made by Benedict (2), McCrudden and Sargent (3), Hunter and Campbell (4), and others. The most serious objection to the method is that when the picric acid filtrate is heated for the transformation of the creatine to creatinine, certain changes apparently take place which yield results that are higher than those obtained when filtrates from other methods of precipitation are similarly treated.

Although Folin and Wu (5) in their system of blood analysis, for obvious reasons of convenience, do not use the picric acid deproteinization, they, nevertheless, still feel that the original process can be utilized when the proper precautions are taken. Because of this controversy and because of the fact that the direct picric acid precipitation of tissue extracts, when creatinine and creatine alone are to be determined, has its elements of convenience in the reduction of dilution and manipulation, a comparison was made of this method and the method of deproteinization with sodium tungstate and  $2/3$  N sulfuric acid.

In this as in the studies to follow, the standards were made from purified creatinine zinc chloride prepared according to Benedict (6). The stock solution was standardized against 0.5 N potassium bichromate and the requisite dilutions made therefrom according to Folin (7). For each series of determinations adequate standard solutions were prepared so that there would be at hand

one with which the unknown solutions would closely correspond. This was considered necessary since the curves of Hunter and Campbell (8), while valid for the conditions under which they worked, may or may not be suitable for use in these studies. The picric acid was purified according to the method of Folin and Doisy (9) and satisfied the requirements stated in their report. Freshly saturated picric acid solutions were always used and were made up in the ratio of 2.5 gm. of the acid to every 100 cc. of distilled water. They were well shaken and the portions that were used for the reaction with the standards were always filtered clear through cotton.

The muscle extracts were prepared from fresh cleaned tissue obtained from the posterior limbs of albino rats killed by ether. The tissue was first ground in a meat chopper, then macerated with an equal weight of fine sand in a mortar, and mixed with an equal volume of Tyrode's solution and 5 cc. of toluene. The suspension was put into a small press and the expressed extract was measured and diluted with an equal volume of Tyrode's solution. 5 cc. portions were used in all the tests.

After several trials the following procedures were developed for the determination of the preformed and total creatinine in the muscle extracts.

In the picric acid deproteinization 5 cc. of the diluted extract are measured into a test-tube or centrifuge tube previously marked at the 15 cc. level, and 10 cc. of a saturated picric acid solution in distilled water are added. After the addition of a small amount of solid picric acid the whole is thoroughly shaken and centrifuged for 3 or 4 minutes. It was found that a better sedimentation is obtained if the contents of the tube are vigorously mixed with a small stirring rod immediately before centrifuging. 10 cc. of the filtrate, obtained by pouring the supernatant fluid in the tube through a bit of cotton in a funnel, are measured into a small flask or vial. 1 cc. of distilled water is added and 1 cc. of 20 per cent sodium hydroxide accurately measured. This ratio between the picric acid and the sodium hydroxide is that used by Folin and Wu (5). The standards for comparison are each made up of 1 cc. of stock creatinine solution containing the appropriate amount of creatinine (0.05 and 0.10 mg. per cc. for the extracts used in these studies), 10 cc. of saturated picric acid, and 1 cc. of

20 per cent sodium hydroxide. Both the unknown and the standard solutions are allowed to stand for 10 minutes and are then compared in the colorimeter. Sometimes it is necessary to filter off through cotton a light flocculent precipitate from the unknown solutions. The addition of the sodium hydroxide should be made with the same pipette and with the same procedure in all cases. It should be noted that in all circumstances the standard solutions are identical with the unknown solutions with respect to the amount of picric acid, and the amount of sodium hydroxide, and are closely similar in colorimetric value.

For the determination of the total creatinine, that is to say, the creatine as creatinine plus the preformed creatinine, 10 cc. of the filtrate from a second 5 cc. sample of extract precipitated as described are put in a small Erlenmeyer flask, diluted with 10 cc. of distilled water, and heated at the boiling point for 2 hours on an electric hot-plate. Partial evaporation is allowable. Complete evaporation is disastrous and is prevented by the addition of small amounts of water from time to time as the occasion demands. During the last half hour of heating the solution may be allowed to concentrate to about 3 or 5 cc. although a 10 cc. final volume does not affect the end-result. The flask is then removed from the hot-plate, cooled to room temperature, and the contents are made to about 16 cc. with distilled water. 1 cc. of 20 per cent sodium hydroxide is added and the mixture is allowed to stand for 10 minutes when it is transferred to a 100 cc. flask and diluted to the mark. The solution so made up is compared with the appropriate standard. It has been found that for extracts prepared as described a standard consisting of 1.5 mg. of creatinine plus 10 cc. of saturated picric acid solution and 1 cc. of 20 per cent sodium hydroxide diluted to 50 cc. after 10 minutes standing is satisfactory.

The final procedure developed for the determination of creatinine and creatine in the tungstic acid deproteinization method is as follows. 5 cc. of the diluted muscle extract are put into a test-tube or centrifuge tube marked at the 15 cc. level, and 5 cc. of distilled water are added. Then 2 cc. each of a 10 per cent solution of sodium tungstate and  $2/3$  N sulfuric acid are added, the whole is made to 15 cc. with water, shaken thoroughly, and centrifuged. For the creatinine determination the supernatant solution

## 130 Creatinine-Creatine in Muscle Extracts. I

is filtered through a bit of cotton and 10 cc. of the filtrate are transferred to a small vial. 10 cc. of saturated picric acid are added and 1 cc. of 20 per cent sodium hydroxide. The standards for these unknown solutions are made by taking 1 cc. of the appropriate original creatinine concentrations, adding 10 cc. saturated picric acid, 9 cc. of distilled water, and 1 cc. of sodium hydroxide. Both standard solutions and unknown solutions are allowed to stand for 10 minutes and are then compared in the colorimeter as previously described. For the determination of the total creatinine, 10 cc. of the filtrate from the tungstic acid precipitation are put into a small Erlenmeyer flask, diluted with 10 cc. of distilled

TABLE I.

*The Amounts of Preformed and Total Creatinine in Muscle Extracts after Deproteinization by Picric Acid and Tungstic Acid.*

Preformed creatinine.		Total creatinine	
Method...	Picric acid.	Picric acid.	Tungstic acid.
mg.	mg.	mg.	mg.
0.125	0.125	7.32	7.32
0.288	0.300	9.89	10.25
0.143	0.112	5.14	4.95
0.120	0.117	5.49	5.40
0.108	0.114	5.49	5.54
0.111	0.114	5.45	5.40
0.071	0.073	5.22	5.22

water, 1 cc. of N hydrochloric acid is added, and the whole is heated for 2 hours as described for the picric acid filtrates, save that in this case the final solution should not exceed 2 or 3 cc. in volume. The flask is removed from the hot-plate, cooled, and 10 cc. of picric acid solution are added and 1 cc. of 20 per cent sodium hydroxide. The mixture is allowed to stand for 10 minutes, is then transferred to a 100 cc. graduated flask, and diluted to the mark. The standard for this determination is exactly the same as that for the analysis of the picric acid filtrate.

When the methods as outlined are carried out on one and the same extract concordant results are obtained as shown in Table I. Parallel determinations were made in all cases.

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## CREATININE AND CREATINE IN MUSCLE EXTRACTS.

### II. THE INFLUENCE OF THE REACTION OF THE MEDIUM ON THE CREATININE-CREATINE BALANCE IN INCUBATED EXTRACTS OF MUSCLE TISSUE OF THE ALBINO RAT.

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The object of this investigation was the determination of the changes that take place in the creatinine and creatine content of extracts of muscle tissue of the albino rat when incubated at body temperature for 24 hours when the reaction of the extract is buffered to neutrality or alkalinity, and when the extract is unbuffered save by the Tyrode's solution used as diluent, and is allowed to develop its own reaction which is slightly acid to rosolic acid. The study is to serve as a foundation for an inquiry into the factors concerned in the creatinine-creatine balance in such tissue extracts with the hope that some light may be thrown on the problems of the metabolism of these compounds.

There are those who have doubted that the demonstration by Stangassinger (1), Gottlieb and Stangassinger (2, 3), Rothmann (4), Mellanby (5), and Myers and Fine (6) of an increase in creatinine accompanied by a decrease in creatine content of muscle tissue or muscle extracts in *in vitro* experiments is a valid indication that such a process occurs in the living organism. The objections raised to such an application of the findings have been based on evidence which has its contradictory phases, while on the other hand, the main facts of the strictly laboratory tests consistently point in one direction. The uniformity of the results of these latter methods of attack can be attributed to the elimination of the interfering factors of digestion, assimilation, utilization, bacterial action, and the probable influence of organs other than the muscles on the reaction being studied. It is not necessary to go into the literature dealing with

this controversy, for to those interested in the problem its main features are well known.

The results of the studies of autolyzed muscle tissue or extracts have shown that the increase of the creatinine content in such preparations occurs whether the reaction of the medium is acid, neutral, or alkaline. But the data are conflicting with respect to the relative influence of the reaction on the amount of creatine formation. The recent report of Hahn and Barkan (7) on the effects of sodium hydroxide and hydrochloric acid on this change in aqueous solutions of creatine while of interest is hardly directly comparable with the studies in which tissue extracts were used.

For the purposes of this study extracts were prepared from the voluntary muscles of the hind limbs of albino rats as described in the preceding paper (8). Rats of the same sex and age were used within each series, although the sex and age differed for the different series. For each series a set of sixteen centrifuge tubes was used and into each tube 5 cc. of muscle extract were measured, using the same pipette throughout. To the first group of four tubes there were added 4 drops of distilled water; to the second group 4 drops of Henderson's (9) phosphate mixture; and to the third 4 drops of a saturated solution of  $\text{Na}_2\text{HPO}_4$ . The remaining four lots were used for the estimation of the preformed and total creatinine of the fresh extract. 0.5 cc. of toluene was added to the above mixtures and they were thoroughly mixed by means of a fine stream of air blown through a glass capillary dipped to the bottom of the tubes. When the reaction of the various groups was tested with rosolic acid, they were found to be slightly acid, neutral, and alkaline, respectively, both before and after incubation. After the contents of the tubes had been prepared as described they were incubated for 24 hours usually at a temperature of  $38^\circ$ , although some lots were kept at  $36$  and  $40^\circ$ . After incubation the preformed and total creatinine were determined according to the picric acid deproteinization method previously described (8). Parallel determinations were made and the reported values represent their averages. The statistical values of the parallel determinations are given in Table I in terms of 0.1 mm. and demonstrate that a considerable degree of reliance can be placed upon the findings.

Turning now to a consideration of the results of these experiments the figures in Table II are given. They represent in mg. the amounts of total and preformed creatinine found in the fresh extracts and in the extracts after incubation under the conditions described. The percentage increase is also tabulated as are the statistical values for the series as a whole. No figures are given for creatine since they are obtained by difference and calculation and would add nothing to the argument.

It is evident that here, as with other workers, there has been an increase in the creatinine content of the extracts on incubation

TABLE I.

*Statistical Values of the Colorimetric Readings of the Parallel Determinations.*

	Mean difference.	Probable error of mean.	Standard deviation.	Probable error of standard deviation.
	0.1 mm.	0.1 mm.	0.1 mm.	0.1 mm.
Creatinine before incubation .....	2.2	0.25	1.76	0.18
Creatinine after incubation.				
Acid .....	2.4	0.31	1.98	0.22
Neutral ... ..	2.4	0.31	1.84	0.22
Alkaline.....	3.0	0.38	2.24	0.27
Creatinine; all determinations....	2.5	0.15	1.97	0.11
Total creatinine before incubation.	2.1	0.25	1.80	0.18
Total creatinine after incubation.				
Acid ... ..	2.2	0.39	2.03	0.28
Neutral .. . . .	3.6	0.49	1.94	0.35
Alkaline .....	2.6	0.46	1.91	0.32
Total creatinine; all determinations.	2.4	0.18	1.94	0.13

whether the reaction of the medium was acid, neutral, or alkaline. This increase is statistically valid as measured by the usual criterion that the probable error of the mean must be contained in the difference between the means at least twice, and three times for definitely satisfactory differences. No changes in the amounts of total creatinine occur on incubation when the same standard of validity is applied. Such being the case and since there has been neither gain nor loss of total creatinine under these conditions, the increase in the creatinine must perforce have been at the expense of the creatine. Since the muscle extracts exhibiting this phenomenon were extracts made with

TABLE II.  
*Changes in the Creatinine-Creatine Balance on Incubation.*

Total creatinine.									
Preformed creatinine.					Fresh.				
	Fresh.		Incubated.			mg.	Incubated.		
			Acid.		Alkaline.		Acid.		Alkaline.
	mg.		mg.	per cent increase	mg.	mg.	mg.	per cent increase	mg.
	0.055	0.134	143.6	0.199	261.8	0.166	201.8	per cent increase	0.6
	0.064	0.167	161.0	0.228	256.2	0.184	187.5	0.4	5.43
	0.067	0.074	10.4	0.094	40.3	0.077	14.9	0.0	3.33
	0.073	0.145	98.6	0.211	189.0	0.163	123.3	-1.8	5.05
	0.074	0.150	102.7	0.188	154.1	0.167	125.9	0.0	4.42
	0.074	0.171	131.1	0.286	286.5	0.214	189.2	-1.1	5.46
	0.078	0.148	89.7	0.185	137.2	0.150	92.3	-0.5	4.50
	0.080	0.135	68.7	0.160	100.0	0.152	90.0	-3.9	4.62
	0.081	0.195	140.7	0.286	253.1	0.212	161.7	0.0	5.53
	0.081	0.213	163.0	0.301	271.6	0.230	184.0	-8.6	5.28
	0.088	0.137	55.7	0.166	88.6	0.157	78.4	-1.3	4.93
	0.116	0.158	36.2	0.188	62.1	0.162	39.6	-0.6	4.83
Mean.....	0.077	0.152	100.0	0.208	175.0	0.169	124.1	-1.5	4.88
Standard deviation.	0.014	0.011	47.8	0.018	85.6	0.012	59.6	2.6	0.58
Probable error of mean .....	0.003	0.002	9.1	0.004	16.7	0.002	11.6	0.5	0.11
Probable error of standard deviation .....	0.002	0.002	6.6	0.003	11.8	0.002	8.2	0.4	0.08
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
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						0.59	0.59	1.4	0.59
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						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
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						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
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						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08
						0.12	0.12	0.3	0.12
						0.59	0.59	1.4	0.59
						0.08	0.08	0.2	0.08

Tyrode's solution which simulates to a considerable degree the medium in which the reactions of the living organism take place, I am of the opinion that we are justified in assuming, until it has been disproved by critical experiment, that there occurs in the muscles of the living organism a formation of creatinine from the muscle creatine and that the endogenous source of the urinary creatinine is the muscle creatine.

This lack of destruction of total creatinine just discussed confirms the findings of Mellanby (5) and Myers and Fine (6) and fails to substantiate the results of Gottlieb and Stangassinger (2). Experiments where putrefaction was allowed to occur, and which will be presented presently, tell another story.

Now when the percentage increase in creatinine is considered it is seen that this increase is regulated in part by the reaction of the medium, for it is least in the acid solutions, greatest in the neutral solutions, and between the two in the alkaline solutions. This relationship is consistently constant in all of the twelve experiments reported and is substantiated by the statistical calculations. It is not in agreement with the result of Rothmann (4), Myers and Fine (6), or Hahn and Barkan (7). For the two former found an apparent acceleration of the reaction by acid and the latter that alkali retarded the change of creatinine to creatine as compared with acid. The studies of Hahn and Barkan (7), however, are hardly comparable with the studies made with tissue extracts. When one looks at the results of Myers and Fine (6) given in Table VII of their paper, it is seen that when the autolyzing mixtures were buffered to neutrality by phosphate mixture a somewhat greater creatinine formation took place than when the tissue was treated with water alone. My results confirm this finding in principle. Nevertheless the studies of Myers and Fine (6) are not strictly comparable with mine inasmuch as they used whole muscle tissue, their periods of autolysis were extended over a longer period and they used an acid not normally found in muscle tissue.

Such being the case it is evident that a slightly acid or an alkaline reaction retards the transformation of creatine to creatinine in muscle extracts when incubated for 24 hours at body temperature. This transformation occurs at a maximum when the reaction of the digesting mixtures is buffered to neutrality by a

phosphate mixture. These facts serve as a partial explanation of the observations of Underhill (10) that creatinuria is frequently an accompaniment of induced acidosis and of Underhill and Baumann (11) that a marked increased creatine excretion may be found in experimental alkalosis, and other apparently anomalous results of the studies of creatinuria, if we admit that the urinary creatinine is largely derived from the muscle creatine, and in spite of the opinions of Denis and Minot (12) and Gamble and Goldschmidt (13) that the acid-base equilibrium has nothing to do with the condition. For since it is shown that both a slight acidity and an alkalinity retard the transformation of creatine to creatinine in muscle extracts it is possible to consider that if similar tendencies are present in the living organism, even though fleeting, they may give rise to similar effects, and if that phase of muscle metabolism which results in creatine formation continues at the same or even a diminished rate, there is produced a relatively greater concentration of creatine in the circulation, part of which at least finds its way to the kidneys and is excreted. That such an increase in blood creatine can occur and continue for days under changed conditions of muscular activity I have already demonstrated (14), though this phase of the problem is not necessarily at present connected with the question of creatinuria and acid-base equilibrium.

Since these experiments demonstrate conclusively that there is no loss of total creatinine on incubation under sterile conditions the figures given in Table III are particularly interesting, from the point of view of the contention of Gottlieb and Stangassinger (3) of the presence in muscle tissue of creatinine—and creatine—destroying enzymes. The results in this table were obtained from extracts which had been allowed to undergo putrefaction during the incubation. It will be seen that there has occurred a marked loss of total creatinine that is statistically valid. This supports the findings of Mellanby (5) that only when bacterial decomposition occurs does there take place a destruction of creatine or creatinine, and plainly shows the cause of the results reported by the proponents of the "creatinase" and "creatase" theory. However, it is quite probable that the transformation of creatine to creatinine in muscle extracts is brought about by an enzyme, in view of the fact that the change occurs in the

TABLE III.  
*Effect of Putrefaction on Changes in Creatinine-Creatine Balance During Incubation.*

Preformed creatinine.										Total creatinine.						
Fresh.	Incubated.									Fresh.	Incubated.					
	Acid.			Neutral.			Alkaline.				mg.	Acid.		Neutral.		Alkaline.
	mg.	per cent increase	per cent increase	mg.	per cent increase	per cent increase	mg.	per cent increase	per cent increase			mg.	per cent increase	per cent increase		
	0.069	0.500	624.9	0.540	682.8	0.509	637.7	5.55	1.99	—64.1	3.00	—45.9	1.28	—76.9		
	0.104	0.544	422.9	0.588	465.5	0.653	527.9	4.48	0.57	—87.3	5.36	0.0	0.83	—81.5		
	0.118	0.300	154.2	0.273	131.4	0.632	435.5	5.36	2.15	—59.9	1.18	—77.2	2.65	—50.6		
	0.121	0.588	386.0	0.741	512.3	0.690	470.1	5.30	1.12	—78.8	1.18	—77.2	0.96	—81.9		
Mean .....	0.103	0.483	396.0	0.555	448.0	0.621	517.8	5.17	1.46	—72.5	3.18	—40.8	1.43	—72.7		
Standard deviation.	0.021	0.111	167.2	0.169	200.1	0.068	76.7	0.41	0.65	11.2	1.71	31.4	0.72	16.7		
Probable error of mean.....	0.007	0.037	56.4	0.057	67.5	0.023	26.0	0.14	0.21	3.8	0.58	10.6	0.24	5.7		
Probable error of standard deviation .....	0.005	0.026	39.8	0.040	47.6	0.016	18.3	0.10	0.16	2.8	0.41	7.5	0.17	4.0		



neutral buffered solutions at a much greater rate than in either the acid or alkaline solutions of muscle extracts or aqueous solutions, or in aqueous solutions uncatalyzed by acid or alkali.

It is interesting to note that the increase of creatinine in these putrefying extracts is much greater than is that which occurs in sterile mixtures. Whether this is a true increase or whether other products are produced by the bacterial action which give the color test for creatinine I am unable to state. The greatest increase takes place in these solutions whose reaction is alkaline. It is also interesting to note that the destruction of the total creatinine occurs about equally well in acid or alkaline media while it is much less in the solutions buffered to neutrality. This may be a direct destruction of creatine or the creatine may first be changed to creatinine which is then destroyed. An explanation of these phenomena is beyond the scope of the present paper, however. The main fact to be gathered is that the only time a destruction of total creatinine is demonstrable is when putrefaction occurs in the incubating extracts.

It should be noted in conclusion that slight differences in temperature during incubation result in differences in amounts of creatinine formation, in that at the lower temperatures the transformation was less. This confirms Myers and Fine (6).

#### SUMMARY AND CONCLUSIONS.

When extracts of muscle tissue of the albino rat are incubated at body temperature for 24 hours there occurs an increase in the creatinine content the relative degree of which depends in part upon the reaction of the incubated extract. When the extract is allowed to develop its own reaction, which is slightly acid to rosolic acid, an increase of 100 per cent takes place. When the extract is buffered to neutrality by phosphate mixture the increase is 175 per cent, and when the extract is made slightly alkaline the increase is 124 per cent. Since there is no change in the total creatinine content of these extracts this increase in creatinine must take place at the expense of the creatine present. Moreover, since the conditions of the experiments simulate to a considerable degree conditions in the living tissue in that the reactions took place in muscle extract diluted with Tyrode's solution it is probable that creatinine is formed from creatine in muscle tissue in the living

organism. The apparent anomaly of an increased creatine excretion in conditions of experimental acidosis and alkalosis is explicable in part on the basis of the retardation of creatinine formation from creatine in incubated muscle extracts when the reaction is slightly acid or alkaline. If similar effects are produced in the organism the continued production of creatine as a result of a phase of muscle metabolism would result in a relatively greater concentration of this in the blood and its excretion in larger amounts in the urine.

It is probable that the transformation of creatine to creatinine in the muscle extracts is facilitated by an enzyme, but no evidence is afforded of the presence in such extracts of any "creatinase" or "creatase." The only time when a destruction of creatinine or creatine takes place is when the extracts undergo putrefaction.

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## STUDIES OF THE THYROID APPARATUS.

### IV. THE INFLUENCE OF PARATHYROID AND THYROID TISSUE ON THE CREATININE-CREATINE BALANCE IN INCUBATED EXTRACTS OF MUSCLE TISSUE OF THE ALBINO RAT.

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The accumulated results of many investigators lead to the idea of a possible relation between parathyroid function, muscle tonus, and creatine-creatinine metabolism.

The occurrence of tetany after removal of the parathyroids and the fact that animals in a state of high neuromuscular tension are more apt to die from acute tetania parathyreopriva after parathyroidectomy than are animals of low tension (1), indicate a connection between parathyroid function and muscle tone. The latter is obviously related to the degree of the activity of the nervous system. The factors concerned in this have been discussed in another place (2).

It is not the purpose of this paper to analyze the controversy concerning the relation of muscle creatine to urinary creatinine which developed from the theory of Folin (3) that urinary creatinine represents a special phase of protein metabolism, probably that of the muscles. It was pointed out in the preceding paper (4) that the present available evidence supports the hypothesis that the source of the urinary creatinine is the muscle creatine. Until such a conception is disproved we shall use it as a working basis for experimentation and interpretation.

The studies on the place of creatine in muscle metabolism which are of interest for our present purpose are those of Weber (5), Cathcart, Henderson, and Paton (6), and Hammett (7). They all indicate that creatine is a by-product of that phase of muscle metabolism concerned in muscle tone. The recent report of Stearns and Lewis (8) on creatinuria in women suggest a relation

between nervous strain with its consequent high neuromuscular tension, and an increased creatine excretion. The relation between muscle tone and creatine-creatinine metabolism is brought out by these observations.

Underhill and Saiki (9), Burns (10), and Greenwald (11) have shown that an increased creatine excretion occurs after the parathyroids have been removed from the experimental animals, and Henderson (12) found an increase in the creatine content of the muscles of parathyroidectomized dogs. This demonstrates a relation between creatine-creatinine metabolism and the parathyroids. This relation may be direct or indirect though the results of these experiments tend to show a direct influence.

This brief outline of the evidence on which the present investigation was begun will suffice as a general statement of the problem. Its points of contact are many. At one point it touches the influence of behavior, in the sense of Paton (13), on metabolism; a problem the importance of which can hardly be over-emphasized.

In view of this relation of parathyroid function, muscle tonus, and creatine-creatinine metabolism it seemed worth while to determine the effect of parathyroid tissue on the changes induced in the creatine-creatinine balance of muscle extracts by incubation for 24 hours at body temperature. In these experiments muscle extracts were made as previously described from muscle tissue of albino rats, and the preformed and total creatinine determinations on the incubated extracts and the fresh material were carried out according to the picric acid deproteinization method discussed in a preceding paper (4). Rats of the same age and sex were used for each individual series but different ages and sexes were used for the different series.

A set of forty centrifuge tubes was used for each series. In all of them there were first put 4 drops of Tyrode's solution. Into each of twelve of them there were then placed three parathyroid glands removed from albino rats immediately after death by ether. Into each of another lot of twelve tubes there were placed three bits of thyroid tissue, each of approximately the same size as a parathyroid gland. Each tube in the thyroid set was comparable to a tube in the parathyroid set since it contained thyroid tissue from the same lobes of the same rats from which the para-

thyroids were taken for its mate. This was done not only for the purpose of exact control, since some thyroid tissue occasionally contaminated the removed parathyroids, but also because it was desired to determine the effect of thyroid tissue on the changes induced by incubation. To the third set of twelve tubes no tissue was added. The remaining four tubes were reserved for use in the determination of the preformed and total creatinine in the fresh extract. There were now added to each of the forty tubes 5 cc. of muscle extract made from the tissues of the same rats from which the parathyroids had been taken.

Since it had been found that the reaction of the incubating extract has a marked influence on the degree of formation of creatinine (4), and since we had no information as to the effect of the reaction on the possible activity of the parathyroids in experiments of this type, it was thought advisable to study the parathyroid and thyroid effects in extracts allowed to develop their own reaction, which is slightly acid to rosolic acid; in extracts buffered to neutrality with Henderson's (14) neutral phosphate mixture; and in extracts made alkaline with a few drops of a saturated solution of  $\text{Na}_2\text{HPO}_4$ . Consequently, there were added to the first four tubes of each of the three sets of twelve, 4 drops of distilled water; to the second four tubes 4 drops of the phosphate mixture; and to the third four tubes 4 drops of the sodium phosphate solution, just as was done in the small series reported in the preceding paper (4). In this way there was obtained a series of rigidly controlled experiments which would show the effects, if any, of the parathyroid and thyroid tissue on the changes in the preformed and total creatinine of muscle extracts in acid, neutral, and alkaline solutions, on incubation for 24 hours at body temperature. Parallel determinations were made throughout. The variability of the parallel determinations was but a little above that found for the experiments made without the addition of parathyroid and thyroid tissue and is consequently not recorded.

The results of these procedures are given in Tables I to IV. In Table I there are recorded the amounts of preformed creatinine in the fresh extract and in the incubated extract with and without the addition of parathyroid tissue in slightly acid, neutral, and alkaline media. The percentage increase in creatinine and the

difference in the percentage increase of the extracts containing the parathyroids from the increase that took place in the controls are also given. Below these values there have been recorded those statistical calculations essential for a determination of the validity of the differences. In Table II the same data are given for the extracts incubated in the presence of thyroid tissue. In Tables III and IV the same data are recorded for the total creatinine with and without the addition of parathyroid and thyroid tissue, respectively.

An inspection of Tables I and II shows that the increased creatinine formation occurs in the controls, in the presence of parathyroid tissue, and in the presence of thyroid tissue; that it is least when the extract is allowed to develop its own reaction, is greatest where the reaction has been buffered to neutrality, and is between the two when an alkaline reaction is maintained. This is a confirmation of the results presented in the preceding paper (4).

From Table I it is evident that the addition of parathyroid tissue to muscle extracts has resulted in a partial inhibition of the formation of creatinine. This retardation occurs in acid and neutral reactions. It occurs to about the same degree in the acid and alkaline extracts, and to a greater degree in the neutral. These differences are statistically valid. From the fact that the maximum tendency to creatinine formation takes place in the extracts buffered to neutrality and the fact that the maximum tendency of the parathyroids to retard this process takes place in solutions of the same reaction, we have almost conclusive evidence of a participation of these glands in creatine-creatinine metabolism. This is further strengthened by the observation that in those extracts to which thyroid tissue was added no such retardation is found that is valid, as can be seen by an inspection of Table II. It is a parathyroid effect, pure and simple. Nor is there any evidence that the addition of the thyroid tissue has any influence whatever on the transformation of creatine to creatinine in muscle extracts.

Turning now to a consideration of the changes induced in the total creatinine by incubation in the presence of parathyroid and thyroid tissue, it is seen from Tables III and IV that while we get a hint of a possible decrease in total creatinine in the acid

TABLE I.  
*The Influence of the Parathyroid Tissue on the Changes in the Performed Creatinine of Muscle Extracts on Incubation.*

Incubated.		Acid.				Neutral.				Alkaline.			
Series.	Fresh.	Parathyroid.		Controls.		Parathyroid.		Controls.		Parathyroid.		Controls.	
		mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease
XVII		0.0810.159	96.30.195	140.7	-44.40.242	198.80.286	253.1	-54.30.207	155.60.212	161.7	-6.1		
XV		0.0550.127	130.90.134	143.6	-12.70.187	239.90.199	261.8	-21.90.159	189.10.166	201.8	-12.7		
XIV		0.0640.137	113.90.167	161.0	-47.10.206	221.90.228	256.2	-34.30.169	164.10.184	187.5	-23.4		
XIII		0.0740.160	116.20.171	131.1	-14.90.241	225.70.286	286.5	-60.80.186	151.30.214	189.2	-37.9		
XII		0.0730.128	75.40.145	98.6	-23.20.194	165.80.211	189.0	-23.20.155	112.40.163	123.3	-10.9		
XI		0.0810.214	164.20.213	163.0	1.20.269	232.10.301	271.6	-39.50.224	176.50.230	184.0	-7.5		
X		0.0880.125	42.00.137	55.7	-13.70.168	90.90.166	88.6	2.30.119	35.20.157	78.4	-43.2		
Mean.....		0.0740.150	105.60.166	127.7	-22.10.215	196.40.240	229.5	-33.10.174	140.60.189	160.8	-20.2		
Standard deviation.....		0.0100.030	36.50.028	35.5	16.60.026	48.90.047	64.2	19.80.032	48.40.027	41.3	13.9		
Probable error of mean.		0.0030.007	9.30.007	9.0	4.20.007	12.50.012	16.3	5.00.008	12.30.007	10.5	3.5		
Probable error of standard deviation....		0.0020.005	6.50.005	6.4	3.00.005	8.80.008	13.3	3.50.006	8.60.005	7.4	2.5		



TABLE II.  
*The Influence of Thyroid Tissue on the Changes in the Performed Creatinine of Muscle Extracts on Incubation.*

Incubated.			Acid.				Neutral.				Alkaline.						
Series.	Fresh.	Thyroid.	Controls.	Differ- ence.	Thyroid.	Controls.	Differ- ence.	Thyroid.	Controls.	Differ- ence.	Thyroid.	Controls.	Differ- ence.				
	mg.	mg.	per cent in- crease	per cent	mg.	per cent in- crease	per cent	mg.	per cent in- crease	per cent	mg.	per cent in- crease	per cent				
XVII		0.081	0.175	116.0	0.195	140.7	-24.7	0.258	218.5	0.286	253.1	-34.6	0.198	144.4	0.212	161.7	-17.3
XV		0.055	0.144	162.0	0.134	143.6	18.4	0.208	278.2	0.199	261.8	16.4	0.165	200.0	0.166	201.8	-1.8
XIV		0.064	0.145	126.6	0.167	161.0	-34.4	0.221	245.3	0.228	256.2	-10.9	0.173	170.3	0.184	187.5	-17.2
XIII		0.074	0.168	127.0	0.171	131.1	-4.1	0.268	262.2	0.286	286.5	-24.3	0.201	172.7	0.214	189.2	-16.5
XII		0.073	0.150	105.5	0.145	98.6	6.9	0.212	190.4	0.211	189.0	1.4	0.158	116.4	0.163	123.3	-6.9
XI		0.081	0.215	165.4	0.213	163.0	2.4	0.296	265.4	0.301	271.6	-6.2	0.226	174.0	0.230	184.0	-5.0
X		0.088	0.127	44.3	0.137	55.7	-11.4	0.183	108.0	0.166	88.6	19.4	0.143	62.5	0.157	78.4	-15.9
Mean.....		0.074	0.161	121.0	0.166	127.7	-6.7	0.235	224.0	0.240	229.5	-5.5	0.181	149.3	0.189	160.8	-11.5
Standard deviation.....		0.010	0.027	37.5	0.028	35.5	17.0	0.037	53.1	0.047	64.2	18.4	0.027	43.4	0.027	41.3	6.2
Probable error of mean.		0.003	0.007	9.6	0.007	9.0	4.3	0.009	13.5	0.012	16.3	4.7	0.007	11.1	0.007	10.5	2.0
Probable error of standard deviation....		0.002	0.005	6.7	0.005	6.4	3.0	0.007	9.5	0.008	13.3	3.3	0.005	7.8	0.005	7.4	1.1

TABLE III.  
*The Changes in the Total Creatinine Content of Muscle Extracts Incubated with Parathyroid Tissue.*

Incubated.			Acid.				Neutral.				Alkaline.						
Series.	Fresh.		Parathyroid.		Controls.		Difference.	Parathyroid.		Controls.		Difference.	Parathyroid.		Controls.		Difference.
		mg.	mg.	per cent in-crease	mg.	per cent in-crease	per cent	mg.	per cent in-crease	mg.	per cent in-crease	per cent	mg.	per cent in-crease	mg.	per cent in-crease	per cent
																</	

Incubated.		Acid.				Neutral.				Alkaline.			
Series.	Fresh.	Thyroid.		Controls.		Thyroid.		Controls.		Thyroid.		Controls.	
	mg.	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease	mg.	per cent in-crease
XVII	5.53	5.44	-1.6	5.53	0.0	5.59	1.1	5.53	0.0	5.49	-0.7	5.49	-0.7
XV	5.19	5.31	2.3	5.20	0.2	5.16	-0.6	5.20	0.2	5.25	1.2	5.22	0.6
XIV	5.41	5.47	1.1	5.43	0.4	5.43	0.4	5.43	0.4	5.34	-1.5	5.43	0.4
XIII	5.55	5.45	-1.8	5.49	-1.1	5.40	-2.7	5.46	-1.6	5.40	-2.7	5.48	-1.3
XII	5.07	5.17	2.0	4.85	-4.4	5.03	-0.8	4.98	-1.8	4.98	-1.8	5.05	-0.4
XI	5.24	5.00	-4.6	4.79	-8.6	5.33	1.7	5.28	0.8	5.24	0.0	5.29	1.0
X	5.06	4.65	-8.1	4.99	-1.4	4.72	-6.7	4.93	-2.6	5.03	-0.6	4.93	-2.6
Mean.....	5.29	5.21	-1.5	5.18	-2.1	5.24	-1.1	5.26	-0.7	5.25	-0.9	5.27	-0.4
Standard deviation.....	0.18	0.28	3.5	0.29	3.0	0.25	2.7	0.22	1.2	0.17	1.2	0.20	1.2
Probable error of mean.	0.05	0.07	0.9	0.07	0.8	0.06	0.7	0.06	0.3	0.04	0.3	0.05	0.3
Probable error of standard deviation.....	0.03	0.05	0.6	0.05	0.5	0.04	0.5	0.04	0.2	0.03	0.2	0.03	0.2

extracts, yet the decreases are too insignificant to justify any conclusion. As a whole the figures make it quite evident that it is impossible to believe that either parathyroid or thyroid tissue have any power to change the total creatinine content of muscle extract treated as described.

These observations fail to substantiate the findings of Rowe (15) that when parathyroid (?) tissue is added to solutions of creatine a decrease of some 30 per cent or more is found to occur. Since Rowe used sheep's thyroid as the source of his destructive agent in the belief that considerable parathyroid tissue is distributed throughout the gland his experiments are really more comparable with those of Table IV, and which fail to show any valid evidence of a loss of total creatinine. In view of his methods of procedure and the great contamination of his material with thyroid tissue, his results can hardly have much bearing on the problem of parathyroid function and creatine-creatinine metabolism.

The fact that an increased creatine excretion and an increased creatine content of muscle tissue follow the loss of the parathyroid secretion is not of itself sufficient evidence that the parathyroids are concerned in creatine metabolism. But when these facts are correlated with the findings presented in this paper that the addition of parathyroid tissue to muscle extracts actually retards the transformation of creatine to creatinine during incubation, we are justified in concluding that the parathyroids are directly concerned in creatine metabolism. The exact rôle of these glands, or the phase of creatine metabolism in which the parathyroids exert their main activity is not as yet clear. Speculation would be premature, particularly in view of the fact of the increased creatine production in the organism when the parathyroids have been removed. The clarification of these points awaits further experimentation.

#### SUMMARY AND CONCLUSIONS.

Evidence is presented which demonstrates that the addition of parathyroid tissue to extracts of muscle tissue of albino rats retards the increase of creatinine formation normally taking place during incubation. This occurs in acid, neutral, or alkaline mixtures. The addition of thyroid tissue to similar extracts has no effect

upon the creatinine formation that is demonstrable by the methods used. Since the maximum retardation effect of the parathyroids occurs in solutions buffered to neutrality, while the maximum creatinine formation takes place at the same reaction, the conclusion is justified that this parathyroid effect is an expression of a direct influence of the parathyroids on creatine metabolism. This conclusion is supported by correlated observations reported in the text.

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## STUDIES OF ACIDOSIS.

### XVII. THE NORMAL AND ABNORMAL VARIATIONS IN THE ACID-BASE BALANCE OF THE BLOOD.

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The possible variations in the acid-base balance of the blood may be stated as follows: the blood bicarbonate may be high, low, or normal, and in each of these conditions the pH may be high, low, or normal. There are as thus classified nine theoretically possible conditions. Only one of them is normal, that in which both bicarbonate and pH are within the normal limits. At the time of the first paper of this series (Van Slyke and Cullen, 1917) only two of the abnormal possibilities had come under clinical observation, that in which bicarbonate is low, and pH normal (compensated acidosis), and that in which bicarbonate is very low, and pH also low (uncompensated acidosis). Now, however, as the result of the recent work of Y. Henderson and Haggard, of Scott, of Milroy, of Collip, of Davies, Haldane, and Kennaway, of Grant and Goldman, of Peters and Barr, and of others, it is known that the other six abnormal possibilities can be produced experimentally, and that at least some of them occur clinically. For this reason it has seemed desirable to enlarge the view presented in our former paper in order to include within it these conditions.

*The Normal and Abnormal Ranges of Hydrion Concentration of the Blood Plasma and Other Extracellular Fluids.*—The average normal hydrion concentration of the blood plasma lies at or near the slightly alkaline point  $H^+ = 4 \times 10^{-8}$ , or  $pH = 7.4$ . This figure was estimated on the basis of material then available by L. J. Henderson in 1909, and has been confirmed by Lunds-gaard (1912), Hasselbalch, and other subsequent investigators, utilizing the gas chain method. Parsons (1917) working with

especial precautions showed that the actual pH value determined is that of the plasma, and that the pH of venous blood in a given individual is normally only 0.02 below that of arterial blood. The maximum normal range of variation of blood reaction in different individuals appears to be indicated by pH 7.30 to 7.50. It is possible that when errors of technique are more completely excluded this range will become still narrower. For a given individual in the resting condition the data of Parsons and of Hasselbalch indicate that the pH variation may be only a few units in the second decimal place.

Under extreme abnormal conditions the pH may fall as low as 6.95, but before this point is reached it appears that coma occurs, and, from the fact that lower pH values have not been observed, it is doubtful that further decrease is compatible with life. This was the lowest point observed by Hasselbalch and Lundsgaard (1912) in rabbits killed by prolonged breathing of air which contained  $\text{CO}_2$ . It was also the lowest point observed by Van Slyke, Austin, and Cullen (1920) in experiments on etherized dogs. A pH of 6.95 was in one instance determined electrometrically by Cullen (unpublished) in the blood of a nephritic man in coma a few hours before death.

By voluntary deep breathing, on the other hand, carbonic acid may be blown off until the blood alkalinity rises to a pH of 7.7 or 7.8 (Davies, Haldane, and Kennaway, 1920; Collip and Backus, 1920), at which point, however, symptoms of tetany appear (Grant and Goldman, 1920). It therefore appears that the extreme range of reaction compatible with life lies approximately between pH 7.0 and 7.8, and that the normal range is within limits no greater than pH 7.3 to 7.5, and possibly somewhat narrower.

Concerning the pH of the body fluids other than blood plasma our knowledge is limited, but such as it is indicates that these fluids approximate the blood plasma closely in their reaction. (By body fluids are meant the fluids within the body proper; such are lymph, cerebrospinal fluid, transudates, exudates, but not secretions such as gastric juice or urine.) Parsons and Shearer (1920) found in the cerebrospinal fluid a pH normal for blood plasma. Cullen and Boots in this Hospital (unpublished data) have observed a similar pH in joint fluids. In other body

fluids, data of Collip and Backus (1920) and of others have shown that the bicarbonate is normal for blood plasma. As there is reason to believe that the  $\text{CO}_2$  tension in these fluids approximates that of the arterial blood (Haggard and Henderson, 1919) it appears that a bicarbonate concentration normal for blood plasma indicates also a  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio, and therefore a pH in these fluids normal for blood plasma (B is used to indicate any monovalent base, such as Na or K,  $[\text{BHCO}_3]$  to indicate the bicarbonate concentration, and  $[\text{H}_2\text{CO}_3]$  the concentration of free carbonic acid).

*The Normal and Abnormal Ranges of the Blood Bicarbonate Concentration.*—Peters and Barr (1921) have reviewed the data in the literature and concluded therefrom that in normal men the total  $\text{CO}_2$  content of the whole blood under 40 mm.  $\text{CO}_2$  tension at  $38^\circ$  falls between 43 and 55 volumes per cent. The  $\text{CO}_2$  content of the plasma is about 8 volumes per cent higher (Joffe and Poulton, 1920). Of the total  $\text{CO}_2$  approximately  $\frac{1}{2}$  is in the form of bicarbonate (Van Slyke and Cullen, 1917). When the  $\text{CO}_2$  tension is other than 40 mm. the normal  $[\text{BHCO}_3]$  figures also change. For when the  $\text{CO}_2$  tension is increased the  $[\text{H}^+]$  is also increased, and the ratio  $(\text{BA}):[\text{HA}]$  for hemoglobin and the other buffers is lowered, since it changes in accordance with the equation  $\frac{[\text{BA}]}{[\text{H}^+]} = \frac{K}{[\text{H}^+]}$  ( $[\text{BA}]$  = concentration of alkali salt of buffer acid, and  $[\text{HA}]$  = concentration of free buffer acid.) In consequence of the change base from the buffers other than bicarbonate is set free and combines with  $\text{CO}_2$  to form  $\text{BHCO}_3$ . Rise of  $\text{CO}_2$  tension therefore increases not only the  $[\text{H}_2\text{CO}_3]$  but also the  $[\text{BHCO}_3]$  and *vice versa*. Consequently above and below 40 mm.  $\text{CO}_2$  tension the normal limits of blood bicarbonate and total  $\text{CO}_2$  content rise and fall as indicated by the two absorption curves of Fig. 1. (These curves are taken from Peters and Barr (1921) who plotted them to include an area containing all the apparently reliable  $\text{CO}_2$  absorption curves of normal human blood in the literature.)

As shown by L. J. Henderson (1921) the variations in the acid-base balance of a given blood are indicated by the changes in any two of a number of interdependent variables, including the buffers other than bicarbonate, the plasma chloride, and the



$[\text{HbO}_2] : [\text{Hb}]$  ratio. In Fig. 1, for example, any point may be located by any two of four values; the total  $\text{CO}_2$ , the  $\text{CO}_2$  tension, the pH, and the  $\text{H}_2\text{CO}_3$ . Of such variables, however, the bicarbonate is of peculiar significance because, as a result of the unlimited supply of  $\text{H}_2\text{CO}_3$ , bicarbonate is the form taken by all bases in the blood not bound by acids other than carbonic. Consequently, as will be seen later, values of  $[\text{BHCO}_3]$ , taken together with those of another determining factor such as the pH, yield information concerning the available alkali which might be difficult to ascertain otherwise.

*Representation of the Combined Variations in Blood Bicarbonate and Hydrion Concentration.*—The blood conditions may be represented by a diagram of the type used by Haldane and others to show the “ $\text{CO}_2$  absorption curves” of the blood, and recently further elaborated by Straub and Meier (1918) and by Haggard and Y. Henderson (1919) to show also the pH values.

If we draw a curve, expressing  $[\text{BHCO}_3]$  values as ordinates, and  $[\text{H}_2\text{CO}_3]$  values as abscissæ, the curve will be a slanting straight line for all points corresponding to any given  $[\text{BHCO}_3] : [\text{H}_2\text{CO}_3]$  ratio, and the slant will be more or less steep according as the  $[\text{BHCO}_3] : [\text{H}_2\text{CO}_3]$  ratio is great or small. But a constant  $[\text{BHCO}_3] : [\text{H}_2\text{CO}_3]$  ratio indicates a constant pH (see equation of Hasselbalch below). Consequently, we are able by a series of straight, slanting lines on a diagram arranged as described to express all possible  $[\text{BHCO}_3] : [\text{H}_2\text{CO}_3]$  ratios and pH values. In pure  $\text{NaHCO}_3 - \text{H}_2\text{CO}_3$  solutions, the isohydronic lines curve slightly, because the proportion of  $\text{NaHCO}_3$  dissociated into  $\text{Na}^+$  and  $\text{HCO}_3'$  increases slightly with dilution. In blood, where the  $\text{Na}^+$  concentration is constant, the lines are practically straight. Their slope may be calculated from the equation of L. J. Henderson (1909),  $[\text{H}^+] = K_1 \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]}$ , or by the same equation in the logarithmic form used by Hasselbalch (1916),  $\text{pH} = \text{p}K_1 + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$ .  $K_1 = \frac{K}{\lambda}$ ,  $K$  being the dissociation constant of carbonic acid,  $\lambda$  the degree of dissociation of  $\text{BHCO}_3$  into  $\text{B}^+$  and  $\text{HCO}_3'$ .  $\text{p}K_1$  is the negative logarithm of  $K_1$ . The value of  $K_1$  for blood was estimated by Haggard and Henderson (1919), from the data available in the literature, as  $8 \times 10^{-8}$ , for which



the negative logarithm, and therefore the corresponding value of  $pK_1$ , is 6.10.

The equation  $pH = 6.10 + \log \frac{[BHCO_3]}{[H_2CO_3]}$  has accordingly been used in plotting the pH lines of Fig. 1 from which Figs. 2 and 3

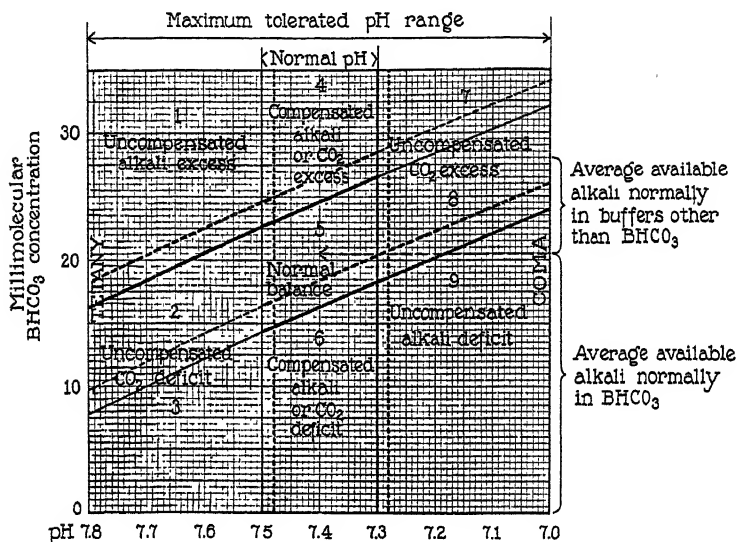


FIG. 2. Normal and abnormal variations of the  $[BHCO_3]$  and pH values in arterial and venous human whole blood. The arterial conditions are indicated by the solid curves, venous by the broken curves.  $[BHCO_3]$  and pH values are represented with rectangular coordinates, and the diagram, as compared with Fig. 1, is simplified by omitting  $[H_2CO_3]$  and  $CO_2$  tension values.  $[BHCO_3]$  values are expressed in terms of millimolecular concentration (1 millimolecular  $[BHCO_3] = 2.24$  volumes per cent of bicarbonate  $CO_2$ ).

are derived. That the value 6.10 will be subject to correction in the second decimal place as the result of further work appears probable, but it is sufficiently accurate to serve our present purposes.

For Fig. 1, since it was desired to use the customary form of  $CO_2$  absorption curves, with total  $CO_2$  values,  $[BHCO_3 + H_2CO_3]$ ,

as ordinates, and  $\text{CO}_2$  tensions as abscissæ, the form of the equation used was  $\text{pH} = 6.10 + \log \frac{(\text{total } \text{CO}_2) - (0.0672p)}{0.0672p}$ ,  $p$  being the  $\text{CO}_2$  tension in mm. of mercury, 0.0672 the factor by which the tension is converted into terms of volumes per cent of  $\text{CO}_2$  physically dissolved (as  $\text{H}_2\text{CO}_3$ ) in the blood (Bohr, 1905).

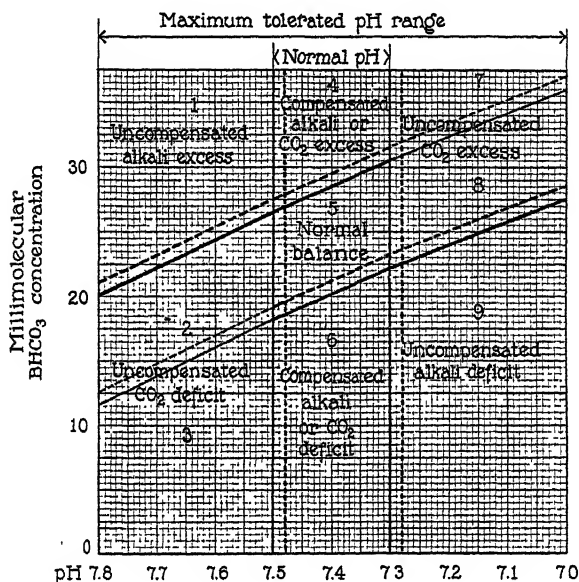


FIG. 3. Normal and abnormal variations of the  $[\text{BHCO}_3]$  and pH in serum or oxalate plasma. Arterial conditions are indicated by the solid curves, venous by the broken curves. The curves are 4 millimols higher than those of Fig. 2, since the  $\text{BHCO}_3$  concentration in the plasma at any given pH is higher than that of the whole blood by approximately 10 volumes per cent of bicarbonate  $\text{CO}_2$ , or 4 millimols of  $\text{BHCO}_3$  per liter. The venous bicarbonate curves are only half as far from the arterial as in the case of whole blood, since the data of Joffe and Poulton (1920), and of Smith, Means, and Woodwell (1921) indicate that the  $[\text{BHCO}_3]$  difference between venous and arterial blood is less in the plasma than in the cells.

*The Combined Variations of Blood Bicarbonate and pH, and the Conditions Associated with Them.*

As stated above, the possible conditions may be classified as those in which the blood bicarbonate is high, low, or normal, and combined with each of these may be a high, low, or normal pH, so that thus classified there are nine possible different conditions of the acid-base balance. These conditions, represented by the nine areas shown in Fig. 1, are the following:

*Area 1.—Uncompensated Alkali Excess.*—In this condition the  $[\text{BHCO}_3]$  is increased above the normal without a parallel increase in  $[\text{H}_2\text{CO}_3]$ . The result is an increase in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and therefore in pH. That is, the blood reaction becomes overalkaline.

The condition appears to occur after overdosing with sodium bicarbonate (Howland and Marriott, 1918; Harrop, 1919; Davies, Haldane, and Kennaway, 1920). It has also resulted from loss of gastric HCl caused by obstructing the pylorus and regularly washing out the stomach for some days (MacCallum, Lintz, Vermilye, Leggett, and Boas, 1920).

It is accompanied by an increase in alveolar  $\text{CO}_2$  tension, due to a slowing of respiration in the apparent attempt of the organism to hold back sufficient  $\text{CO}_2$  to restore to normal the overalkaline reaction. (If this compensation is accomplished the condition shifts to that represented by Area 4.) There is a moderate diuresis, and bicarbonate is excreted in the urine at a rate that may be several grams per hour (Davies, Haldane, and Kennaway). Ammonia almost completely disappears from the urine and the titratable acid may become a negative quantity. The cessation of acid excretion and its replacement by alkali excretion tend to reduce the  $[\text{BHCO}_3]$  to normal, and bring the system back, probably through Area 4, to the normal condition represented by Area 5.

The most marked and characteristic clinical effect of uncompensated alkali excess is the development of symptoms of tetany when the alkalinization proceeds sufficiently far. From this fact, however, one is not at present justified in assuming that all tetany is either caused by, or accompanied by, alkalosis. McCallum and his coworkers did not find high plasma bicarbonate

in tetany caused by parathyroidectomy, although McCann (1918) did find it.

*Areas 2 and 3.—Uncompensated CO<sub>2</sub> Deficit.*—In this condition the  $[\text{H}_2\text{CO}_3]$  is decreased without a parallel fall in  $[\text{BHCO}_3]$ . The result is, therefore, as in the condition represented by Area 1, an increase in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH, but it is due to loss of  $[\text{H}_2\text{CO}_3]$  instead of increase in  $[\text{BHCO}_3]$ .

Area 2 represents the first result of lowering in blood  $[\text{H}_2\text{CO}_3]$  by a respiratory stimulus other than either the blood hydron or  $\text{H}_2\text{CO}_3$  concentration. The consequence is an overalkaline reaction.

A compensating retention of acid metabolites, indicated by a decreased excretion of ammonia and titratable acid in the urine sets in, and there is also, as when the pH is raised by increased  $[\text{BHCO}_3]$ , an excretion of bicarbonate (Davies, Haldane, and Kennaway). As a result of these compensatory processes the bicarbonate of the blood may be lowered in some hours from Area 2 to Area 3 (partial compensation), and eventually to Area 6, where the pH is again down to its normal value (entire compensation). This last condition is attained when one becomes acclimated to a high altitude (Hasselbalch and Lindhard, 1915).

Uncompensated CO<sub>2</sub> deficit has been caused in man by hyperpnea either voluntary (Collip and Backus, 1920; Davies, Haldane, and Kennaway, 1920; Grant and Goldman, 1920; Milroy, 1914), or induced by breathing air with a diminished oxygen content, such as is encountered at high altitudes (Haggard and Henderson, 1920; Haldane, Kellas, and Kennaway, 1919). Bazett and Haldane (1921) have observed it as the result of hyperpnea caused by emersion in warm water. Apparently unusual demands on the lungs for either oxygenation or cooling may arouse respiratory stimuli which to some extent rob the hydron stimulus of its usual control.

The effects of uncompensated CO<sub>2</sub> deficit on the urine are similar qualitatively to those of uncompensated alkali excess; there is a decrease in ammonia excretion, an increase in urinary pH, and an excretion of bicarbonate (Davies, Haldane, and Kennaway). The rate of bicarbonate excretion observed, however, was much less (only a fraction of a gram per hour) when the blood pH was raised by overbreathing than when it was raised by administration of bicarbonate.

The ultimate clinical symptoms are again those of tetany

and have been identified as such by Grant and Goldman (1920). The characteristic signs after voluntary deep breathing for an hour or less included the carpopedal spasm, Chvostek's sign, Trousseau's sign, Erb's sign, and in one instance even a tetanic convulsion. The physiological effects of abnormally high blood pH appear to be similar, whether the increase is caused by an increase in the numerator or a fall in the denominator of the  $[\text{BHCO}_3]: [\text{H}_2\text{CO}_3]$  ratio.

Area 4.—Compensated  $\left\{ \begin{array}{l} \text{Alkali Excess or} \\ \text{CO}_2 \text{ Excess.} \end{array} \right.$ —Here the pH is normal,

the  $[\text{BHCO}_3]$  is high but is balanced by a proportionally high  $[\text{H}_2\text{CO}_3]$ . The state of the acid-base balance of the blood is the same, whether the original disturbance is alkali retention (Area 1) or  $\text{CO}_2$  retention (Areas 7 and 8). Hence the condition may be described as either compensated alkali excess or compensated  $\text{CO}_2$  excess, according to whether the primary disturbance is due to alkali or  $\text{CO}_2$  retention. The condition indicated by Area 4 has been observed to arise from both causes.

*Alkali excess* has been observed after therapeutic overadministration of sodium bicarbonate. If, as is usually the case following moderate oral administration, the absorption is not rapid,  $\text{CO}_2$  may be retained sufficiently to balance the increased  $[\text{BHCO}_3]$ , and the condition changed from that indicated by Area 5 merely to that of Area 4. If absorption is too rapid for simultaneous compensation by  $\text{CO}_2$  retention, the condition changes to that indicated by Area 1, presumably to return later through Area 4 to normal Area 5.

*Compensated  $\text{CO}_2$  excess* appears to be the state observed by Scott (1920) in emphysema. The retarded gas exchange presumably leads to a state of chronically increased  $\text{CO}_2$  tension in the blood, and the body raises the blood  $[\text{BHCO}_3]$  high enough to balance the  $[\text{H}_2\text{CO}_3]$  and maintain a normal reaction.

It appears that this condition, primarily due to  $\text{CO}_2$  retention, may be differentiated from that in which alkali retention is the primary cause, by the fact that the former is associated with cyanosis (as in emphysema), either permanent or caused by slight exertion. Diffusion of oxygen is so much slower than that of  $\text{CO}_2$  (Krogh, 1919) that any hindrance retarding the alveolar gas exchange sufficiently to affect  $\text{CO}_2$  excretion would presumably be accompanied by still more hindrance to oxygenation of the

blood. This presumption is further supported by the work of Krogh and Krogh (1910) who found in rabbits that while  $\text{CO}_2$  tension in arterial blood and alveolar air are equal, oxygen tension is lower in the blood than in the alveolar air, even when respiration is unhindered.

*Area 5.—Normal Acid-Base Balance.*—The normal area represents the balance that is practically always found in the resting individual in health and at ordinary altitudes. (At higher altitudes the normal dissociation curve falls parallel with the barometric pressure (Y. Henderson, 1920)). Area 5 covers approximately the conditions represented in detail by the nomogram of L. J. Henderson (1921).

The minimum and the maximum normal arterial  $\text{CO}_2$  tensions and bicarbonate concentrations indicated by the lower left and upper right corners of Area 5 are about twice as far from the means as are the normal extremes for these values heretofore estimated from alveolar air and arterial blood analyses. The wide range indicated by the diagram may be due to the fact that technical errors have widened in all directions the ranges indicated by the boundaries of Area 5. More accurate data will perhaps show this area to be smaller, and the extremes therefore less far apart. For the pH limits of the plasma Parsons is inclined to place the normal range at more nearly between pH 7.30 and 7.40 than the doubly wide range of 7.30 to 7.50 which we have allowed.

Another reason in part perhaps responsible for the fact that the extreme  $[\text{BHCO}_3]$  and  $\text{CO}_2$  tension values of Area 5 exceed the normally observed extremes is that there would be only one chance out of many for maximum pH and minimum  $[\text{BHCO}_3]$ , or the reverse, to occur in the same individual; *e. g.*, if levels of each so far from the mean are taken as to include only 1 individual out of 20, presumably only 1 out of 400 would show both extremes at once. Consequently it would not be surprising if the extreme normal limits of  $\text{CO}_2$  tension and  $[\text{BHCO}_3]$  have hitherto escaped observation, or have been observed so rarely as not to be regarded normal.

With more accurate technique and a larger number of observations it appears probable that the limits of normal arterial  $\text{CO}_2$  tension and bicarbonate concentration indicated by a graphic estimation like the above will coincide with those observed.



Area 6.—Compensated  $\left\{ \begin{array}{l} \text{Alkali Deficit or} \\ \text{CO}_2 \text{ Deficit.} \end{array} \right.$  —Area 6 represents a

condition in which the available blood alkali is lowered, but in which a normal pH is maintained because the fall in  $[\text{BHCO}_3]$  is balanced by a proportional fall in  $[\text{H}_2\text{CO}_3]$ . The primary cause of the condition may be a fall in either  $[\text{H}_2\text{CO}_3]$  or  $[\text{BHCO}_3]$ , decrease in the other factor being in each case a secondary balancing or compensatory process, with the apparent physiological object of maintaining a normal pH. Although the final state of the acid-base balance in the blood is in each case the same, *viz.* proportionally lowered  $[\text{BHCO}_3]$  and  $[\text{H}_2\text{CO}_3]$  with normal pH, the state may nevertheless with some advantage be differentiated by two terms, as either compensated alkali deficit or compensated  $\text{CO}_2$  deficit, according to whether the primary cause of the abnormality is deficit in the available alkali, caused by retention or overrapid formation of non-volatile acids, or whether it is deficit in  $[\text{H}_2\text{CO}_3]$  caused by some respiratory stimulus added to or intensifying the usual  $[\text{H}^+]$  stimulus. In alkali deficit the result of a failure in the secondary compensating processes would be acidification, in  $\text{CO}_2$  deficit it would be alkalinization. Presumably different means, depending on the nature of the primary cause, may be required in each case to restore and maintain normality.<sup>1</sup>

*Compensated alkali deficit* is the condition occurring as the result of accelerated production of non-volatile acids (diabetes) or their retarded elimination (presumably the case in nephritis). The bicarbonate reserve of the entire body is diminished, that of the blood falling parallel with that of the other body fluids. In experimental intoxication of rabbits with  $\text{HCl}$ , Goto (1918) has found that the potassium phosphate of the tissues and the  $\text{CaCO}_3$  of the bones are also reduced.

In the evident attempt to maintain a normal  $[\text{H}_2\text{CO}_3]:[\text{BHCO}_3]$  ratio and pH in the blood, ventilation becomes deeper, and the  $[\text{H}_2\text{CO}_3]$  is reduced in proportion to the  $[\text{BHCO}_3]$ . Presumably there is during the acid invasion a slight increase in hydron concentration, causing the blood condition to shift toward the border which separates Areas 6 and 9. The respiratory center is, however, at once stimulated, and  $\text{CO}_2$  is driven off so

<sup>1</sup> Whether the lowered blood alkali of traumatic shock is primarily due to non-volatile acid retention (Cannon, 1918) or to  $\text{CO}_2$  loss (Y. Henderson and Haggard, 1918) is still uncertain.

that until compensation breaks down pH is kept within normal limits and the condition remains in Area 6. At the same time accelerated formation of ammonia and excretion of buffer acids, such as acid phosphate, tend to raise the available alkali back to normal.

Compensated alkali deficit is the condition commonly observed as the result of retention of non-volatile acids in metabolic diseases, such as diabetes and nephritis (discussed by Van Slyke and Cullen, 1917) and marasmus of infants (Schloss and Harrington, 1919; Howland and Marriott, 1916). Until recently it has been the only form of naturally occurring acidosis recognized clinically, except the uncompensated acidosis (Area 9) of the premortal state. Since the pH is normal, the alkali neutralized by the invading acids is solely that of the bicarbonate (see page 169), and the fall in blood bicarbonate is an exact measure of the non-volatile acid that enters the blood.

For the reason that this condition at the time represented all clinically observed acidoses, except the uncompensated premortal acidosis, in which also, however, the bicarbonate is reduced, Van Slyke and Cullen in 1917 defined acidosis as a condition in which the blood bicarbonate is lowered. The definition is adequate for the forms of acidosis caused by retention of non-volatile acids, but it does not cover the conditions since observed which are represented by Areas 7 and 8, and which are caused by CO<sub>2</sub> retention; and it fails to exclude the conditions represented by Areas 2 and 3, in which the bicarbonate is reduced as a result not of acid retention but of CO<sub>2</sub> loss. With apparent adequacy, however, one may define acidosis as a condition caused by acid retention sufficient to lower either the bicarbonate or the pH of the blood below the normal limits.

*Compensated CO<sub>2</sub> Deficit.*—As already stated above, a fall of blood alkali with maintenance of normal pH may also occur when the primary cause is not acid retention, but excessive respiratory loss of CO<sub>2</sub>. In this case the fall in blood bicarbonate is a compensatory process which tends to prevent the blood reaction from becoming abnormally alkaline. One respiratory stimulant which has been demonstrated to have such an effect is oxygen want.

Y. Henderson (1920) has shown from data obtained by Fitzgerald, and by Douglas, Haldane, Henderson, and Schneider

(1912) on their Pike's Peak expedition that the  $\text{CO}_2$  of the alveolar air is lowered at high altitudes, and varies in direct proportion to the barometric pressure. This is also shown by the data of Hasselbalch and Lindhard (1915). Since the rate of  $\text{CO}_2$  production is not lowered (Hasselbalch and Lindhard), it is evident that the minute volume of air breathed is increased, an effect which is also noted when air with reduced oxygen percentage is breathed at sea-level pressure.

The process by which the state of compensated  $\text{CO}_2$  deficit is reached has been outlined in the discussion of the condition represented by Areas 2 and 3.

The final effect, lowered bicarbonate with normal pH, is the same as in compensated retention of non-volatile acids. The primary cause, however, is not acid retention, but loss of an acid (carbonic) which is compensated by a reduction of the blood alkali.

*Areas 7 and 8.—Uncompensated  $\text{CO}_2$  Excess.*—In this condition respiratory excretion of  $\text{CO}_2$  is retarded, either by physical hindrance or by deadening of the respiratory center, so that the  $[\text{H}_2\text{CO}_3]$  of the blood is raised. In consequence the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH are lowered. The actual blood reaction becomes less alkaline than normal.

This condition has been caused experimentally by breathing air which contains 3 to 5 per cent of  $\text{CO}_2$  (Hasselbalch and Lunds-gaard, 1912; Davies, Haldane, and Kennaway, 1920). It appears to be caused also when the respiratory center is deadened by morphine narcosis (Michaelis and Davidoff, 1912; Henderson and Haggard, 1918).

Means, Bock, and Woodwell (1921) report an observation of the condition in a cyanotic pneumonia patient.

The physiological effects are seen in an accelerated excretion of ammonia and titratable acid by the urine, the same as when the acid-base balance is shifted towards the acid side by retention of non-volatile acids. Davies, Haldane, and Kennaway (1920) observed a doubling of the rate of ammonia and titratable acid excretion after breathing air containing up to 5 per cent of  $\text{CO}_2$ . There is also, as in non-volatile acid retention, an increase in the minute volume of air expired in the apparent attempt to get rid of the excess of  $\text{CO}_2$ , unless the respiratory center is deadened, as by morphine.

The first effect of  $\text{CO}_2$  retention on the blood is to increase the  $[\text{H}_2\text{CO}_3]$  and  $[\text{H}^+]$  of the blood, without changing the buffer alkali content (condition represented by Area 8). Thus Davies, Haldane, and Kennaway found after breathing for an hour air containing  $\text{CO}_2$  in amounts gradually increasing up to 5 per cent, there was no change in the  $\text{CO}_2$  capacity of the blood (unchanged total buffer alkali). Henderson and Haggard (1918) found in dogs a rise of 2 or more volumes per cent in  $\text{CO}_2$  capacity of the blood within a half hour after injecting morphine, or breathing air containing 5 or more per cent of  $\text{CO}_2$ .

The increase in the blood alkali occurring in Area 7 is secondary to the  $[\text{H}_2\text{CO}_3]$  increase and is compensatory in its nature; it tends to raise the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio back to normal by increasing the  $[\text{BHCO}_3]$  to balance the increased  $[\text{H}_2\text{CO}_3]$ . In consequence the blood condition from Area 8 shifts to Area 7 (partial compensation). Normal Area 5 is presumably regained either by passing directly back to Area 5 from Area 8 (in case the excess  $\text{CO}_2$  is blown off before secondary rise of alkali to Area 7 occurs), or by passing through Areas 7 and 4 to 5 (in case compensation is accomplished partly by the secondary rise in alkali).

The compensatory increment of blood alkali in Area 7 probably comes from two sources: (1) The increased excretion of ammonia and titratable acid through the kidneys tends to raise the bicarbonate content of the entire body, and the blood plasma bicarbonate would normally rise with that of the other fluids. (2)  $\text{HCl}$  may perhaps leave the blood plasma and enter the tissue cells, as it has been shown to leave the plasma and enter the blood cells when the pH rises (for discussion and literature on this electrolyte shift see Van Slyke, 1921). The rate of blood alkali rise observed by Henderson and Haggard appears too rapid to be probably accounted for by acid excretion alone, and these authors attribute the increase to alkali drawn from the tissues. The effect would be the same if acid passes from the blood into the tissues, a process which from analogy with the shift between plasma and blood cells seems more probable. The relative parts that these two factors, acid excretion and shift of acid to the tissues (or of alkali in the reverse direction), play in the compensatory rise of blood bicarbonate during  $\text{CO}_2$  retention is uncertain. That accelerated acid excretion occurs has been shown. That acid shift from blood to tissues also occurs seems probable.

*Area 9.—Uncompensated Alkali Deficit.*—In this condition the  $[\text{BHCO}_3]$  of the blood is lowered without a proportional fall in  $[\text{H}_2\text{CO}_3]$ . In consequence there is a fall in the  $[\text{BHCO}_3]:[\text{H}_2\text{CO}_3]$  ratio and the pH.

This is the condition defined by Hasselbalch and Gammeltoft (1915) as "uncompensated acidosis." It has been most frequently observed in cases of nephritic and diabetic acidosis in the premortal period. Means, Bock, and Woodwell (1921) describe both the symptoms and blood changes in such a nephritic case. The blood bicarbonate is extremely low. Respiration, which up to the terminal stage has kept the  $[\text{H}_2\text{CO}_3]$  sufficiently low to maintain a normal  $[\text{H}_2\text{CO}_3]:[\text{BHCO}_3]$  ratio, now fails to do so, and the blood condition shifts from that represented by Area 6 over into that represented by Area 9.

In deep ether anesthesia, according to the results of Van Slyke, Austin, and Cullen (1919-20), the blood state is represented by Area 9, and both alkali deficit and carbonic acid retention occur. This combination appears also to occur in some cardiac cases (Peters and Barr, 1921).

*Relation of Changes in the Acid-Base Balance of the Blood to  
Changes in the Other Body Fluids.*

As has already been shown, the intercellular fluids other than blood plasma have, so far as studied, been found under normal conditions to approximate the blood plasma in bicarbonate and hydron concentrations. There is evidence that in changes from the normal the other body fluids follow more or less promptly the blood plasma. Van Slyke and Cullen (1917) found that when acid was injected into the circulation the fall in blood bicarbonate was only about one-sixth as great as it would have been had the acid all remained in the blood; the other five-sixths of the acid must have gone into the other body fluids and the tissues, or drawn alkali from them. Palmer and Van Slyke (1917) found similarly that when bicarbonate was administered the rise in blood bicarbonate was approximately that calculated on the assumption that the alkali was not retained in the blood, but was distributed evenly through all the body fluids. Collip and Backus (1920) found that the bicarbonate of the spinal fluid follows that of the venous blood plasma. When the latter was lowered by

continued etherization, or by shock (handling of intestines) the bicarbonate of the spinal fluid also fell and to about the same level, although more slowly. When the bicarbonate of the blood was raised by bicarbonate injection, the spinal fluid bicarbonate rose in the course of a few hours to approximately the same level.

*The Physiologically Available Alkali of the Bicarbonate and of the Other Blood Buffers.*

In order that a buffer shall neutralize an acid (HCl for example) *without change in pH* it is necessary that the buffer acid HA, set free by the reaction  $BA + HCl = BCl + HA$ , shall be completely removed, and in addition as much of the HA formerly present as may be necessary to keep the ratio  $[BA]:[HA]$  at the original value. Of the blood's important buffers, plasma protein, cell phosphates, hemoglobin, and bicarbonate, only the bicarbonate has an acid which can be quickly removed. Under nearly all circumstances, physiological and pathological, in which the respiratory apparatus is not specifically affected, it appears that the  $[H_2CO_3]$  is so regulated that a normal pH is maintained. This is accomplished even by the ill diabetic or nephritic so efficiently that, until the  $[BHCO_3]$  has been reduced by invading acids to one-fourth, and perhaps even one-eighth of its normal value, the  $[H_2CO_3]$  is reduced in the same proportion, and a normal pH is maintained.

So long as the pH is kept constant in the above manner, all the changes in buffer alkali are those of the bicarbonate. For at a constant pH the ratio  $[BA]:[HA]$  remains constant for each buffer, in accordance with the general equation for salts of weak acids; *viz.*,  $\frac{[HA]}{[BA]} = K_1 [H^+]$ . Consequently, however much depletion the  $[BHCO_3]$  may suffer, the alkali salts of the other buffers are unaffected.

However, when a fall in pH occurs, the alkali of the other buffers is drawn upon and, as mentioned before, conditions have been recently observed in which a fall in pH does occur. From the data available it appears that for a short time at least the pH may fall as low as 7.0, although not much lower without fatal results.

The maximum available alkali of the blood is therefore almost the entire alkali of the bicarbonate plus that portion of the other buffer alkalies which is yielded when the pH changes from normal to the minimum compatible with life. The amount of available buffer alkali may be estimated by increasing the  $\text{CO}_2$  tension of the blood until the pH is reduced from 7.4 to 7.0. All the alkali given up by the other buffers is bound by  $\text{H}_2\text{CO}_3$  and thereby turned into bicarbonate under these conditions, so that the increase in bicarbonate above that at normal  $\text{CO}_2$  tension and pH 7.4 represents the available alkali of the other buffers. By extrapolation of the average normal  $\text{CO}_2$  absorption curve of human blood in Fig. 2 we find that this increase covers the range from 20.5 to 28.0 millimolecular  $[\text{HCO}_3^-]$ . The available alkali from buffers other than bicarbonate is therefore 7.5 millimolecular in concentration, equivalent to  $2.24 \times 7.5 = 17$  volumes per cent of  $\text{CO}_2$ , or approximately one-third the normal blood bicarbonate alkali.

The average total alkali of normal human blood available for neutralizing invading acids may therefore be summarized as:

0.0205 M bicarbonate alkali, (equivalent to 46 volumes per cent of bicarbonate  $\text{CO}_2$ ). Of this three-fourths, and perhaps seven-eighths, may be used for neutralization of acid without change in pH and most of the remainder becomes available if the pH falls to 7.0.

0.0075 M alkali, (equivalent to 17 volumes per cent of bicarbonate  $\text{CO}_2$ ), from other buffers, available only when the pH falls to 7.0.

Total 0.280 M available alkali, (equivalent to 63 volumes per cent of bicarbonate  $\text{CO}_2$ ).

Of the 0.0075 M alkali available from buffers other than bicarbonate the greater part is normally bound to hemoglobin (Van Slyke, 1921)<sup>2,3</sup>

<sup>2</sup> p. 160.

<sup>3</sup> If the amounts of alkali bound to oxyhemoglobin at pH 7.4 and 7.0 are calculated by the equation,  $\frac{[\text{BHCO}_3]}{[\text{HHCO}_3]} = \text{pH} - \text{pK}$ , placing  $[\text{BHCO}_3] + [\text{HHCO}_3]$  equal to 0.045 M and pK, for hemoglobin equal to 7.2 (Van Slyke, 1921), we calculate in fact that the oxyhemoglobin in normal blood would yield 0.010 M alkali in changing from pH 7.4 to 7.0. This is  $\frac{1}{3}$  more than all the buffers together yield according to the curves of Fig. 2. The quantitative discrepancy will presumably disappear when the constants for hemoglobin are more accurately determined, and perhaps also when the normal  $\text{CO}_2$  absorption curves in the pH range 7.3 to 7.0 are more accurately worked out.

In a broad sense, therefore, the alkali reserve of the blood includes not only the bicarbonate, but in addition about one-third as much alkali from the other blood buffers. In a still broader sense one might add the alkali of the bicarbonate and other buffers in the tissues and body fluids outside the circulation, since it also becomes available when the blood is flooded with acid (Van Slyke and Cullen, 1917).<sup>4</sup> In the sense, however, that it contains the only alkali that can neutralize acids without fall in blood pH, the bicarbonate forms a reserve in a class by itself.

*Means for Determining State of the Acid-Base Balance.*—It is not the purpose of the present paper to discuss in detail the technique for studying the acid-base balance of the blood and the body, but the principles appear derivable from the preceding discussion.

In order to determine which one of the possible variations exists in the blood *in vivo* it is necessary to ascertain two of the involved variables, such as the pH,  $[\text{BHCO}_3]$ , and  $[\text{H}_2\text{CO}_3]$ . With any two of them a point can be located in its proper area on a diagram such as Fig. 1, 2, or 3, but with any one of them alone it cannot be done.<sup>5</sup>

Under most conditions, pathological as well as normal, the pH is kept normal. When this is the case determination of either the  $\text{CO}_2$  tension or the bicarbonate in either plasma or whole blood suffices to indicate the condition. The plasma bicarbonate determination by the gasometric method of Van Slyke and Cullen (1917) or the titration method of Van Slyke, Stillman, and Cullen (1919) is adequate for this case, and therefore suffices for the study of metabolic conditions (*e.g.* those usually met in

<sup>4</sup> p. 338.

<sup>5</sup>When the blood is only partially saturated with oxygen, accurate location of its point on a diagram such as Fig. 1, based on figures from complete oxygenated blood, will involve a correction for the oxygen unsaturation. (The effect of oxygenation and reduction of hemoglobin in the blood bicarbonate was discovered by Christiansen, Douglas, and Haldane (1914) and has been discussed theoretically by L. J. Henderson (1920) and by the writer (1921).) For a diagram such as Fig. 1, with  $\text{CO}_2$  contents as ordinates and  $\text{CO}_2$  tensions as abscissæ, Peters and Barr (1921) have estimated the approximate correction as  $-0.34$  volume per cent of  $\text{CO}_2$  for each volume per cent of oxygen unsaturation.



diabetes, nephritis, and metabolic disturbances of infants) in which the source of acid-base disturbance is retention of non-volatile acids, while the respiratory control of the blood reaction is unaffected. It is not adequate to cover conditions in which the respiratory control of the blood reaction is so disturbed that the pH becomes abnormal, as happens in anesthesia.

Joffe and Poulton (1920) and Peters and Barr (1921) have suggested, as the preferable single blood determination, the  $\text{CO}_2$  content of the whole blood determined after equilibration with air containing  $\text{CO}_2$  at 40 mm. tension. This estimation is sufficient to indicate whether the available alkali is normal or abnormal, but to indicate the entire state of the acid-base balance it is inadequate. Thus from reference to Fig. 1 it is evident that a  $\text{CO}_2$  capacity of 30 volumes per cent determined at 40 mm.  $\text{CO}_2$  tension on whole blood indicates a bicarbonate reserve about 12 volumes per cent (in  $\text{CO}_2$  terms) below the minimum normal. Whether the condition existing in the body is that indicated by Area 3, 6, or 9, is, however, left uncertain.

In regard to the use of such diagrams as have been employed in this paper a conclusion indicated by results of Hasselbalch, of Parsons, and of Peters and Barr may be reiterated; *viz.*, that in order to draw the most accurate deductions concerning the variations in the acid-base balance of a given individual it may be necessary to know the conditions that are normal for his particular blood. For each individual Area 5 of our diagram presumably shrinks to a fraction of the area, indicated on the charts in this paper, required to include the normal variations of the species.

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## PREPARATION AND ANALYSIS OF ANIMAL NUCLEIC ACID.

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In recent years a number of publications have appeared dealing with the problem of animal nucleic acids. The greatest part of the work was done by Steudel and by Feulgen, and more recently there appeared a publication by Thannhauser. The work from the Berlin laboratory was done during the years of the war and became accessible to us in original form only recently.

Some of the work of Feulgen<sup>1</sup> and the recent publication of Thannhauser deal with the problem of the mode of linking of the nucleotides in the tetranucleotide. Our tentative view on this part of the problem is expressed in another article, and we shall not refer to it here.

Other articles deal with the problem of the occurrence of animal nucleic acids of two new types. The acids of one type contain only three instead of the four nucleotides, thus the new type of acids from this view-point are not tetra- but trinucleotides. It was claimed to have been isolated from the pancreas gland. On the other hand Feulgen claimed to have isolated a more complex nucleic acid containing besides the hexose nucleotides also a pentose nucleotide; namely, guanylic acid. We shall refer to these acids as mixed nucleic acids. The evidence adduced by Feulgen and later by Hammarsten<sup>2</sup> in favor of the existence of mixed nucleic acids did not seem to us convincing.

<sup>1</sup> Feulgen, R., *Z. physiol. Chem.*, 1913, lxxxviii, 370; 1917, c, 241; 1917-18, ci, 288. Feulgen, R., and Landmann, G., *Z. physiol. Chem.*, 1918, cii, 262. Feulgen, R., *Z. Physiol. Chem.*, 1919-20, cviii, 147.

<sup>2</sup> Hammarsten, E., *Z. physiol. Chem.*, 1920, cix, 141.

Also the evidence in favor of the existence of a trinucleotide in the pancreas seemed to us to require further corroboration.

Recently we were forced to resume the preparation of animal nucleic acid required for our work since the technical production of the substance in Europe has been discontinued. This necessity gave us the occasion to repeat some of our older work, with the result that we have improved somewhat our older method of preparation of the nucleic acids and the method of analysis of the purine bases in nucleic acids. In the main the method of preparation is the same as previously used by us; the new details are given in the experimental part.

For the analysis of the purine bases it was found convenient to employ alcoholysis instead of hydrolysis.

If nucleic acid is suspended in methyl alcohol and hydrochloric acid gas is passed through the solution the nucleic acid is rapidly dissolved and soon the purine bases begin to separate in the form of their hydrochloric acid salts. The separation is practically completed in 2 hours. The heat developed by the absorption of the gas is sufficient to bring about the desired cleavage.

The bases are colored very little and are readily isolated for analysis. The acids were obtained from three organs: spleen, pancreas, and liver. These three organs were selected for the reason that they are all known to contain guanylic acid together with the animal nucleic acid.

From the spleen nucleic acid, the present writer obtained a nucleic acid<sup>3,4</sup> which contained the bases in the following proportions; adenin 8.17 per cent, guanin 9.15 per cent, cytidin 7.0 per cent, and thymin 8.0 per cent. The theory for a tetra-nucleotide requires, respectively, 9.56, 10.72, 7.86, and 8.93 per cent. In a recent paper, Steudel<sup>5</sup> writes that the presence of a true nucleic acid in the spleen is still in need of proof. His method of proving it is not more rigorous than the one employed by the previous writer.

The substances now prepared had the following composition:

<sup>3</sup> Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 370.

<sup>4</sup> Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 215.

<sup>5</sup> Steudel, H., *Z. physiol. Chem.*, 1921, cxiv, 255.

	C	H	N	P
Spleen nucleic acid.....	36.15	4.07	15.52	9.71
Pancreas " ".....	36.20	4.76	15.26	9.58
" " ".....	35.07	5.43	14.95	9.59
Liver " ".....	36.75	4.65	11.65	10.60
" " ".....	37.08	4.96	11.51	10.31
Theory for a hexosetetranucleotide....	36.30	4.19	14.79	8.73

None of the samples showed the presence of pentose and all, even the samples from the liver, contained the two purine bases. The yield of the bases was smaller, however, from the pancreas and liver nucleic acids. They were as follows:

From:	Adenin picrate.	Crude guanin.
	<i>gm.</i>	<i>gm</i>
Spleen nucleic acid.....	16.0	10.0
Pancreas " ".....	9.0	7.5
Liver " ".....	8.0	7.6

It is obvious that the elementary composition of amorphous substances of the nature of nucleic acids, which are never free from impurities, is of comparatively little service for the purpose of forming theories of molecular structure. However, great deviations from the theory, such as are observed on liver nucleic acid, require an explanation. Also, the fact that the pancreas nucleic acid, which in its elementary composition does not differ much from the spleen nucleic acid and yet furnishes on hydrolysis less purine bases than the other, needs further explanation. Work on these problems is now in progress.

#### EXPERIMENTAL.

*Preparation of Nucleic Acids.*—This is uniform for all three of the tissues, and the details of the procedure are as follows:

2,500 gm. of minced fresh tissue (previously freed from fat) are taken up in 3,000 cc. of water. 300 gm. of sodium chloride are added and all is kept boiling (with a steam coil) for 4 hours. Then 80 gm. of sodium acetate and 60 cc. of a 33 per cent solution of sodium hydroxide are added, and the mixture is allowed to



stand over night. The mixture is then neutralized with acetic acid and treated with picric as long as a precipitate forms. To the filtrate of this mixture hydrochloric acid is added until it turns slightly opalescent and the nucleic acid is precipitated with a 10 per cent solution of copper chloride. The copper salt of the nucleic acid is filtered and converted into the free acid by treatment with a 5 per cent solution of hydrochloric acid. The treatment is repeated once. The resulting free nucleic acid is redissolved in a 5 per cent solution of sodium hydroxide, the solution is made acid with acetic acid and precipitated with 95 per cent alcohol, containing 4 per cent of hydrochloric acid. The precipitate is then washed with 95 per cent alcohol until the washing no longer shows the presence of chlorine ions. The nucleic acid is then washed with absolute alcohol and ether, and dried. The substance so obtained does not show the presence of even traces of biuret-giving substances, and gives a negative test with orcin.

#### *Alcohololysis.*

10 gm. of nucleic acid are suspended in 200 cc. of dry methyl alcohol and hydrochloric acid gas is passed in a lively stream. After 2 hours the reaction is interrupted and the flask is allowed to stand over night. Guanin and adenin are separated in the usual way.

Guanin was purified by conversion into the sulfate, which again was converted into the free base. Adenin picrate for purification was twice recrystallized out of 10 per cent acetic acid and then dissolved in water by the addition of the required quantity of ammonia and precipitated by means of acetic acid.

#### *Analysis of Nucleic Acids.*

##### *Spleen Nucleic Acid.*

0.9880 gm. of the substance gave on combustion 0.1310 gm. of  $\text{CO}_2$  and 0.0360 gm. of  $\text{H}_2\text{O}$ .

0.0884 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 9.8 cc. of 0.1 N acid.

0.1768 gm. of the substance gave 0.0616 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	Calculated for $C_{15}H_{19}N_{15}P_4O_{32}$ . per cent	Found. per cent
C.....	36.30	36.15
H.....	4.19	4.07
N.....	14.79	15.52
P.....	8.83	9.71

*Bases.*—10.0 gm. of this material yield 1.6 gm. of adenin picrate and 1.0 gm. of guanin. The guanin was analyzed as the free base.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required 66.30 cc. of 0.1 N acid for neutralization.

	Calculated for $C_8H_5N_5O$ . per cent	Found per cent
N.....	46.53	46.41

The adenin picrate analyzed as follows:

0.0946 gm. of the dry substance employed for Kjeldahl nitrogen estimation (reduced with zinc metal) required for neutralization 21 cc. of 0.1 N acid.

	Calculated for $C_8H_5N_5.C_6H_5(NO_2)_3OH$ . per cent	Found. per cent
N.....	30.71	31.06

### *Pancreas Nucleic Acid.*

A. 0.1056 gm. of the dry substance on combustion gave 0.1358 gm. of  $CO_2$  and 0.0572 gm. of  $H_2O$ .

B. 0.1080 gm. of the dry substance of a second sample gave 0.1434 gm. of  $CO_2$  and 0.0460 gm. of  $H_2O$ .

A. 0.1818 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 19.40 cc. of 0.1 N acid.

B. 0.1780 gm. of another sample required for neutralization 19.40 cc. of 0.1 N acid.

A. 0.2726 gm. of the substance gave 0.0938 gm. of  $Mg_2P_2O_7$ .

B. 0.2670 gm. of another sample gave 0.0918 gm. of  $Mg_2P_2O_7$ .

	Calculated for $C_{15}H_{19}N_{15}P_4O_{32}$ . per cent	Found. per cent	
		A	B
C.....	36.30	35.07	36.02
H.....	4.19	5.43	4.76
N.....	14.79	14.95	15.26
P.....	8.73	9.59	9.58

*Bases.*—The substance was alcoholized in 10.0 gm. lots. The average yield was 0.900 gm. of adenin picrate and 0.750 gm. of crude guanin. Guanin was analyzed as the free base and gave the following values.

0.1066 gm. of the substance gave 0.1566 gm. of  $\text{CO}_2$  and 0.0310 gm. of  $\text{H}_2\text{O}$ .

0.0996 gm. of the substance gave 39.4 gm. nitrogen gas at  $T = 26^\circ$  and  $P = 756$  mm.

	Calculated for $\text{C}_8\text{H}_5\text{N}_5\text{O}$ . per cent	Found. per cent
C.....	39.73	39.05
H.....	3.30	3.26
N.....	46.60	46.62

Adenin picrate analyzed as follows:

0.0935 gm. of substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.25 cc. of 0.1 N acid.

	Calculated for $\text{C}_8\text{H}_5\text{N}_5\text{O} \cdot \text{C}_6\text{H}_3(\text{NO}_2)_3\text{OH}$ . per cent	Found. per cent
N.....	30.71	30.32

### *Liver Nucleic Acid.*

A. 0.9720 gm. of the dry substance gave 0.1330 gm. of  $\text{CO}_2$  and 0.0434 gm. of  $\text{H}_2\text{O}$ .

B. 0.1090 gm. of another sample gave 0.1390 gm. of  $\text{CO}_2$  and 0.0566 gm. of  $\text{H}_2\text{O}$ .

A. 0.0900 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 7.40 cc. of 0.1 N acid.

B. 0.1816 gm. of another sample required for neutralization 13.60 cc. of 0.1 N acid.

A. 0.1800 gm. of the dry substance gave 0.0666 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

B. 0.2724 gm. of another sample gave 0.1108 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	Calculated for $\text{C}_{42}\text{H}_{32}\text{N}_{12}\text{P}_2\text{O}_{12}$ per cent	Found. per cent	
		A	B
C.....	36.30	37.08	34.79
H.....	4.19	4.96	5.81
N.....	14.79	11.51	10.48
P.....	8.73	10.31	11.33

*Bases.*—One lot of 50.0 gm. was treated in methyl alcohol and hydrochloric acid gas as described above. This yield was 3.8 gm. of crude guanin and 4.0 gm. of crude adenin picrate.

Guanin was identified as the free base and analyzed as follows:

0.0997 gm. of the base gave on combustion 0.1566 gm. of  $\text{CO}_2$  and 0.0338 gm. of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_5\text{H}_5\text{N}_5\text{O}$ . <i>per cent</i>	Found. <i>per cent</i>
C.....	39.73	39.55
H.....	3.30	3.47

Adenin was identified as picrate and analyzed as follows:

0.0940 gm. of the substance employed for nitrogen estimation (modified Kjeldahl) required for neutralization 20.50 cc. of 0.1 N acid.

	Calculated for $\text{C}_5\text{H}_5\text{N}_5$ $\text{C}_6\text{H}_3(\text{NO}_2)_3\text{OK}$ . <i>per cent</i>	Found. <i>per cent</i>
N.....	30.71	30.53

unsaturation of either one of the two acids is as yet not known. There are however, indications that one (arachidic) may be tetra-unsaturated. On the addition of bromine a substance was obtained which analyzed for an octobromide of arachidic acid. However, it will require a larger quantity of material to establish the degree of unsaturation of each of the two acids with certainty. The presence of several acids in the liver lecithin again emphasizes the question of the existence of more than one lecithin.

It was attempted to answer this question by the molecular weight estimation of the hydrolecithin. The hydrolecithin from the liver lecithin has been prepared essentially according to Paal's procedure.

The molecular weight of the substance was found 810 and 700 (in two estimations). The theory of a monophosphatide requires 809, that of a diphosphatide 1600. Consequently, liver lecithin consists of a mixture of monolecithins.

In the course of the present work the process of preparation of pure free lecithin from its cadmium chloride salt has been improved so that analytically pure substance is prepared in good yield; namely, about 50 gm. of free lecithin from 100 gm. of the cadmium chloride salt.

The procedure in the main is as follows: The salt of lecithin is dissolved in chloroform and this solution is transferred into a solution of dry ammonia gas in dry methyl alcohol. The resulting lecithin is purified from the slight quantity of impurities by the acetic acid process developed by Levene and Ingvaldsen. The details of the procedure are given in the experimental part.

#### EXPERIMENTAL.

##### *I. Preparation of Pure Lecithin.*

Various attempts to produce pure liver lecithin without converting it into the cadmium chloride salt met with little success. The following method proved to be the easiest and most efficient.

The liver, in 100 pound lots, is minced, dried, and extracted, first with acetone, second with ether, and last with alcohol. These extracts are treated separately as follows:

*Acetone Extract.*—This is allowed to stand at 0°C. over night. A precipitate of fat is deposited, which is removed by filtration.

The filtrate is concentrated (if necessary) and the lecithin precipitated by adding a saturated solution of cadmium chloride in alcohol until no further precipitate is formed.

The residue from the above filtration is suspended in alcohol and warmed until the fat is melted. The mixture is then cooled over night. The fat precipitated on standing is again filtered from the alcoholic solution and again treated with alcohol as before. This extraction is repeated until the mother liquor no longer gives with cadmium chloride a precipitate of lecithin cadmium salt. The latter is recognized by the fact that on dissolving in a small amount of moist ether it is again precipitated by the addition of an excess of acetone. From three to seven extractions may be required. The alcoholic mother liquors are then precipitated with cadmium chloride.

*Ether Extract.*—This is concentrated to a small volume and allowed to stand at 0°C., when a precipitate consisting of fat and cerebrosides is formed. The precipitate is extracted with ether. The ethereal extract is added to the original filtrate and cooled once more to permit the separation of the cerebrosides which the solution may still contain. After filtering, the combined mother liquors are concentrated and treated with alcohol to separate the lecithin from cephalin. The alcoholic liquors are then treated with cadmium chloride.

*Alcoholic Extract.*—This is likewise concentrated and cooled to remove cerebrosides, the mother liquor being decanted if possible, otherwise filtered, or centrifuged if necessary.

The cerebrosides are again extracted with warm ether. The extract is cooled and centrifuged. The alcoholic and ethereal liquors are then treated with cadmium chloride.

#### *Treatment of Cadmium Chloride Salts.*

It is necessary to allow the cadmium chloride precipitate of lecithin to stand at least half an hour until it is sufficiently coagulated to permit filtration. The filtered material, which is not quite dry, is transferred to a large beaker or precipitating jar and stirred up with a large volume of cold acetone. If the acetone liquor turns dark from dissolved material the suspension is allowed to settle, the liquor decanted off, and more cold acetone is added. Finally the material is filtered by suction.

This material is purified in two steps: the one, is the "ether crystallization," the other is the "toluene-ether" process.<sup>1</sup> It is a matter of judgment as to which shall be used first and the number of times which each should be repeated. The aim is to obtain a white granular material which filters quickly.

*The ether crystallization* consists in dissolving the cadmium chloride salt in warm ether, water being added, a few drops at a time, until the suspended material goes into solution. An excess of water hinders the solution of the larger particles. The solution is allowed to stand over night, or longer, at 0°C. The substance should separate in a granular form, easily filterable by suction. If it forms a pasty solution not easily filtered, time and material will be wasted in attempting a filtration. Another precipitation with acetone should remove impurities which interfere with the process.

This purification removes not only the fats and oils but also takes out most of the cephalin present. Since the cadmium chloride salt is itself slightly soluble in cold ether, some of the material may be lost in purification. Hence the following precautions are necessary. 1. Excess of ether is to be avoided. With very impure material it is more advisable to repeat the purification several times with small quantities of solvent than to use a large excess at one time. The amount of ether filtered off should not be more than twice the volume of the residue. 2. The filtered material should *not* be washed with ether, but should be filtered as quickly as possible until the solvent runs very slowly. 3. In case the material fails to filter properly, it should be transferred to a beaker, warmed slightly until dissolved, precipitated with acetone, purified by the toluene-ether method, and subsequently passed through the ether crystallization process. 4. The filtration should be carried out in the cold.

*The toluene-ether* process consists in dissolving the cadmium chloride salt in a minimum volume of toluene (adding a slight amount of water if necessary). If the toluene fails to dissolve all the material the residue should be centrifuged off. The solution is then treated with 4 volumes of ether containing 1 per cent water. The solution is cooled to 0°C. over night and filtered.

The latter method gives larger yields but removes less of the cephalin and other impurities. It probably removes impurities not taken out by the former method, hence the cadmium chloride compound should be purified by both methods.

Experience shows that in the case of liver lecithin the toluene-ether method should precede the ether crystallization method of purification of the cadmium chloride salt in order to obviate difficulty in filtering from the ether.

One purification by each method should be sufficient to give almost white dry material with an amino content of less than 3 per cent of the total nitrogen present. Such a product may be converted into free lecithin.

#### *Conversion of the Cadmium Chloride Compound into Free Lecithin.*

The cadmium chloride salt is dissolved in chloroform and is converted into free lecithin by means of a solution of ammonia in methyl alcohol. 100 gm. of the cadmium chloride salt are dissolved in 300 cc. of warm chloroform and poured into 400 cc. of methyl alcohol containing 20 gm. of ammonia gas. This is added slowly with rapid stirring. The product of reaction is allowed to stand a short time before filtering. The precipitate may be filtered off through a folded filter paper. The chloroform methyl alcohol solution of lecithin is then concentrated under diminished pressure. Near the end of the concentration the material foams considerably for a short time and then the foaming subsides. The vacuum concentration should be carried out at a low temperature. If during the operation a precipitate of fat settles out this should be filtered off. The remaining lecithin is practically free from solvent. It is dissolved in a minimum (5 to 10 cc.) of glacial acetic acid. This is poured into 800 cc. of boiling hot acetone, stirred, and allowed to cool to room temperature. A very small dark precipitate (1 to 2 gm.) settles out. The supernatant liquid is decanted or filtered. The precipitate is slightly soluble in ether and insoluble in acetone but somewhat soluble in ethyl alcohol and more soluble in methyl alcohol.

No. 126.

0.0154 gm. of substance gave on combustion 0.0954 gm. of  $H_2O$ , 0.2315 gm. of  $CO_2$ , and 0.0114 gm. of ash.



0.1910 gm. of substance used for Kjeldahl nitrogen determination required 3.90 cc. of 0.1 N acid corresponding to 0.00546 gm. of N.

0.2865 gm. of substance gave 0.0390 gm. of  $Mg_2P_2O_7$ .

$C_{44}H_{87}O_9NP$ . Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 61.16, H 10.34, N 2.92, P 3.88.

It contains 10 per cent amino nitrogen.

No. 124.

1.5 gm. of substance were hydrolyzed with HCl, neutralized, concentrated, and made up to 15 cc.

5 cc. of this solution required for Kjeldahl nitrogen determination 5.60 cc. of 0.1 N HCl.

2 cc. of this solution for Van Slyke determination gave 0.57 cc. of  $N_2$  at  $T = 27^\circ$ ,  $P = 762.2$  mm.

$$\frac{\text{Amino N}}{\text{Total N}} = \frac{10}{100}$$

The liquors are then cooled in a freezing mixture to  $-5^\circ C$ . Frequently at this phase a second small precipitate settles out. A sample of this material analyzed as follows:

No. 122.

0.1024 gm. of substance gave on combustion 0.1098 gm. of  $H_2O$ , 0.2216 gm. of  $CO_2$ , and 0.0118 gm. of ash.

0.1832 gm. of substance for Kjeldahl nitrogen determination required 3.10 cc. of 0.1 N acid corresponding to 0.00434 gm. of N.

0.2748 gm. of substance gave 0.0464 gm. of  $Mg_2P_2O_7$ .

$C_{44}H_{87}O_9NP$ . Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 59.50, H 12.10, N 2.37, P 4.75.

The mother liquors are concentrated under diminished pressure until all the ether and most of the acetic acid are removed. Water is added a little at a time and the material is shaken or stirred until a thick emulsion of a light brown color is formed. This is poured into 800 to 1,000 cc. of acetone, chilled down to  $-5^\circ C$ . It is carefully stirred and allowed to stand at 0 to  $-5^\circ C$ . over night, when it is transferred to a crystallizing dish and washed free from excess water by stirring with cold dry acetone. The acetone is decanted off and the lecithin dried in a vacuum desiccator.

From 40 to 45 gm. of pure material may be obtained from 100 gm. of cadmium chloride salt. (Theoretical yield, 81 to 82 gm.) Several samples have been analyzed. They differed little one from another in their elementary composition. The analysis of one of them is as follows:

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of  $H_2O$ , 0.2768 gm. of  $CO_2$ , and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of  $Mg_3P_2O_7$ .

$C_{44}H_{87}O_9NP$ . \* Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 64.83, H 11.16, N 1.87, P 4.03.

\* This formula represents material consisting of equal parts of two lecithins, each one containing two of the four fatty acids.

## II. The Fatty Acids of Lecithin.

For the preparation of fatty acids from lecithin, the material was hydrolyzed 8 to 15 hours with 10 parts of 10 per cent HCl. The fatty acids on cooling appeared as a semisolid cake. They were dissolved in methyl alcohol and precipitated in the presence of ammonium hydroxide with a nearly equal weight of lead acetate dissolved in a minimum quantity of water. After freezing, the mother liquors were filtered off. The lead salts which contained both the saturated and unsaturated fatty acids were extracted repeatedly with boiling ether until further extraction produced only slight precipitate with hydrochloric acid.

The ether solution then contained the lead salts of the unsaturated acids while the ether-insoluble material consisted of the lead salts of the saturated acids. Both fractions were decomposed with HCl, dissolved in ether, washed with water, dried, and the solvent evaporated off.

A lot of 528 gm. of the lecithin cadmium chloride free from amino nitrogen was hydrolyzed with 10 per cent solution of hydrochloric acid. The yield of fatty acids was 223 gm.

### *Unsaturated Fatty Acids.*

These were obtained by extracting the lead salt first by means of acetone and then by means of ether. Each extract was worked over separately. The acetone extract was concentrated and the residue thus obtained extracted with ether. From both of these fractions the acids were liberated and reconverted into the lead salts. These were again purified and again converted into the free acids. A sample of the acids gave the following iodine and hydrogen values.

0.2907 gm. of substance absorbed 0.435 gm. of iodine by the Wijs method.  
 0.5141 gm. of substance reduced by Paal's method absorbed 67 cc. of  $H_2$  in 3 hours at  $17^\circ C.$ , 759 mm. pressure, or 1.103 gm. of  $H_2$  per 100 gm. of substance.

$C_{18}H_{34}O_2$ .	Calculated.	Iodine value	91,	Hydrogen number	0.721.
$C_{18}H_{32}O_2$ .	Calculated.	" "	182,	" "	1.447.
	Found.	" "	154,	" "	1.103.

It was later found that this material consisted of two fatty acids, hence it is possible that one was a singly unsaturated, and that the other contained two or more double bonds.

The free fatty acids were finally reduced by Paal's method. The samples of reduced acids obtained from each fraction analyzed as follows:

No. 84 (material obtained from the acetone extract of the lead salts).

0.1020 gm. of substance gave on combustion 0.1198 gm. of  $H_2O$  and 0.2830 gm. of  $CO_2$ .

No. 85 (material obtained from the ether extract of the lead salts).

0.1012 gm. of substance gave on combustion 0.1190 gm. of  $H_2O$  and 0.2828 gm. of  $CO_2$ .

No. 84. C 75.96, H 13.19.

" 85. C 76.21, H 13.15.

Since the two fractions proved practically of identical elementary composition they were combined and converted into the methyl esters. These were freed from adhering sulfuric acid by washing with water and finally by recrystallization from methyl alcohol. They were then fractionated by distilling at a pressure of 1 to 2 mm.

The following fractions were obtained.

A.....	182-185°C.
B.....	175-185°C.
C.....	182-195°C.
D.....	185-203°C.

Fractions A and D were redistilled and the following fractions were obtained.

From A	A <sub>1</sub> .....	158-165°C.
	A <sub>2</sub> .....	170-182°C.
From D	D <sub>1</sub> .....	182-192°C.
	D <sub>2</sub> .....	187-197°C.

For identification, the esters were saponified with an alcoholic solution of sodium hydroxide. The acids were liberated and converted into the lead salts. The acids were again liberated from the lead salts and analyzed. Fraction A<sub>1</sub> corresponded apparently to pure stearic acid.

Analysis 101.

0.1024 gm. of substance gave on combustion 0.1186 gm. of H<sub>2</sub>O and 0.2860 gm. of CO<sub>2</sub>.

0.8950 gm. of substance in a molecular weight determination required 6.50 cc. of 0.5 N NaOH.

C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. Calculated. C 75.93, H 12.76.

Found. C 76.16, H 12.96.

Molecular weight was 275, that of stearic acid is 284.

The substance melted at 70.5–71°C., stearic acid melts at 70–71°C.

When this was mixed with some very pure stearic acid melting at 74°C., the mixture melted at 74°C.

Fraction D<sub>2</sub> apparently corresponded to pure arachidic acid.

Analysis 100.

0.1000 gm. of substance gave on combustion 0.1166 gm. of H<sub>2</sub>O and 0.2822 gm. of CO<sub>2</sub>.

0.9760 gm. of substance neutralized 6.75 cc. of 0.5 N NaOH.

C<sub>20</sub>H<sub>40</sub>O<sub>2</sub>. Calculated. C 76.95, H 12.91.

Found. C 76.97, H 13.24.

Molecular weight was 314, that of arachidic acid is 313.

The substance melted at 75.5–76°C., arachidic acid melts at 75–77°C.

When this was mixed with some pure arachidic acid melting at 75°C., the mixture melted at 75°C.

*Saturated Fatty Acids.*

The lead salts which were insoluble in acetone and ether were converted into free acids. These were twice esterified with methyl alcohol. The mixture of methyl esters thus obtained was distilled at a pressure of 1 to 2 mm. into the following fractions:

a.....	160–163°C.
b.....	159–167°C.
c.....	158–172°C.
d.....	170–180°C.

and residue.

Fractions a and d were redistilled as follows.

From a	a <sub>1</sub> .....	156-162°C.
	a <sub>2</sub> .....	Residue.
From d	d <sub>1</sub> .....	180-183°C.
	d <sub>2</sub> .....	182-188°C.

Fraction a<sub>1</sub> apparently corresponded to pure palmitic acid.

Analysis 92.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H<sub>2</sub>O and 0.2802 gm. of CO<sub>2</sub>.

0.8168 gm. of substance neutralized 6.14 gm. of 0.5 N NaOH.

C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>. Calculated. C 74.92, H 12.58.

Found. C 75.09, H 12.98.

Molecular weight was found to be 266, palmitic acid had a molecular weight of 256.

The melting point was 62°C., palmitic acid melts at 63-64°C.

When this was mixed with some pure palmitic acid melting at 64°C., the mixture melted at 63°C.

Fraction d<sub>2</sub> apparently corresponded to pure stearic acid.

Analysis 94.

0.1009 gm. of substance gave on combustion 0.1220 gm. of H<sub>2</sub>O and 0.2802 gm. of CO<sub>2</sub>.

0.6686 gm. of substance neutralized 4.82 cc. of 0.5 N NaOH.

C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. Calculated. C 75.93, H 12.76.

Found. C 75.72, H 13.53.

Molecular weight was 278, stearic acid had a molecular weight of 284.

The substance melted at 71°C., stearic acid melts at 70-71°C.

When this was mixed with a sample of very pure stearic acid melting at 74°C. the mixture melted at 74°C.

### III. Bromine Addition Products of the Unsaturated Acids.

An attempt was made to separate and to characterize the individual unsaturated acids by preparing the bromine addition products. 40 gm. of pure lecithin which had been prepared from the cadmium chloride salt as described above, were used.

No. 119.

0.0996 gm. of substance gave on combustion 0.0994 gm. of H<sub>2</sub>O, 0.2768 gm. of CO<sub>2</sub>, and 0.0090 gm. of ash.

0.1798 gm. of substance required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2697 gm. of substance gave 0.0390 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

C<sub>44</sub>H<sub>87</sub>O<sub>9</sub>NP. Calculated. C 65.59, H 10.89, N 1.74, P 3.86.

Found. C 64.83, H 11.16, N 1.87, P 4.03.

This was hydrolyzed with a 10 per cent solution of hydrochloric acid, the acids were dissolved in ether, washed with water, dried, and the ether evaporated off. The iodine number of the mixed acids was 91.

0.2457 gm. of substance absorbed 0.232 gm. of iodine by the Wijs method.  
Average molecular weight of 280. Calculated. Iodine value 91.  
Found. " " 91.

The acids were converted into the lead salts, the unsaturated acids extracted with ether and converted into the free acids. These were dissolved in 18–30° petrolic ether and brominated at 0°C. with 3 cc. of bromine dissolved in petrolic ether.

On freezing to  $-10^{\circ}$  a precipitate was obtained. The mother liquor was concentrated and again cooled to  $-10^{\circ}$ . The combined precipitate was recrystallized from petrolic ether and then recrystallized from ethyl ether.

This gives three fractions: A, the petrolic ether-soluble fraction; B, the fraction insoluble in petrolic ether but soluble in ethyl ether; and C, the fraction insoluble in both solvents. This last fraction contains the material having most bromine (namely, the hexabromides and octobromides, if present). The first fraction should be largely dibromides while the tetrabromides should predominate in the fraction insoluble in petrolic ether but soluble in ethyl ether.

Fraction C (the material insoluble in both solvents) was recrystallized from ethyl ether; the yield was 1 gm. In an open tube melting point determination it darkened, turning black at 200°C. It contracted at 240°C. and decomposed at 243°C. In a closed tube it contracted at 239°C. and melted without decomposition at 243°C. This analyzed as follows:

No. 129.

0.2012 gm. of substance gave 0.2936 gm. of AgBr.

This would indicate a hexabromide.

$C_{20}H_{34}O_2Br_6$	Calculated for hexabromarachidic acid.	61.0.
$C_{18}H_{30}O_2Br_6$	Calculated for hexabromstearic acid.	63.2.
Found.		62.11.

The material was recrystallized from ether. On heating in an open tube it darkened at 180–200°C., contracted at 240°C., and decomposed at 244°C.

It analyzed as follows:

Analysis 130.

0.1068 gm. of substance gave 0.1670 gm. of AgBr.

This corresponds more closely to an octobromide.

$C_{20}H_{32}O_2Br_8$ .	Calculated for octobromarachidic acid.	67.80.
	Found.	66.55.

There was not sufficient material for further treatment.

#### IV. *Hydrolecithin from Liver Lecithin.*

For the preparation of hydrolecithin 10 gm. of pure liver lecithin free from amino nitrogen were used (Analysis 119 given above). This was reduced by Paal's method. The hydrolecithin produced was recrystallized twice from acetone and once from methyl ethyl ketone.

This analyzed as follows:

No. 128.

0.1074 gm. of substance gave on combustion 0.1082 gm. of  $H_2O$ , 0.2554 gm. of  $CO_2$ , and 0.0098 gm. of ash.

0.1926 gm. of substance for Kjeldahl nitrogen determination required 2.40 cc. of 0.1 N acid, corresponding to 0.00336 gm. of N.

0.2839 gm. of substance gave 0.0400 gm. of  $Mg_2P_2O_7$ .

$C_{44}H_{91}O_9NP$ .	Calculated.	C 65.30, H 11.33, N 1.73, P 3.84.
	Found.	C 65.03, H 11.29, N 1.74, P 3.86.

A molecular weight determination was made as follows:

1.036 gm. of substance raised the boiling point of 16 gm. methyl alcohol  $0.071^\circ C$ .

0.964 gm. of substance raised the boiling point of 16 gm. methyl alcohol  $0.077^\circ C$ .

$C_{44}H_{91}O_9NP$ .	Calculated.	Molecular weight 809.
	Found. First determination	810.
	Second " "	700.

# ON THE NUMERICAL VALUES OF THE OPTICAL ROTATIONS IN THE SUGAR ACIDS.

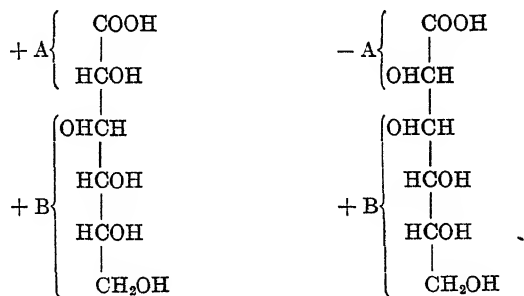
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In recent years the van't Hoff theory of optical superposition has been on several occasions the subject of discussion. Some writers, Rosanoff, and independently of him Patterson and Taylor have challenged the theory, whereas in a series of very important publications Hudson<sup>1</sup> has demonstrated the validity of the theory and made it the foundation of many important contributions to the chemistry of carbohydrates.

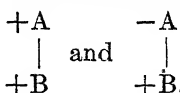
The present writer also has made use of the theory for the purpose of establishing relationships between the configuration of the carbon atom 2 and the optical rotation of epimeric sugar acids. It was found that in a pair of epimeric sugar acids as in gluconic and mannonic acids the molecular structure may be regarded as consisting of two parts, one (A) consisting of carbon atoms 1 and 2, and the other (B) of carbon atoms 3, 4, 5, and 6 as seen from the following.



<sup>1</sup> Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66; 1917, xxxix, 462; 1918, xl, 813.



It is evident that such pairs of epimers may be represented as



and It was found that in sugar acids and in

2-hexosaminic acids the superposition theory holds to the following extent; the direction of the rotation of the carbon atom 2 is in agreement with the theory in all of the four pairs of hexonic and all of the four pairs of hexosaminic acids. The numerical value of the rotation of carbon atom 2 should according to the theory of van't Hoff be identical for all hexonic and for all hexosaminic acids. This expectation was not realized. In three of the epimers of each group the values are identical, but in the fourth it is markedly different as seen from the following table.

	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{20}$	Phenylhy- drazides of	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{20}$
Epichitosaminic ..	+12.5	+24.37 (10 <sup>2</sup> )	Gluconic....	+14.25	+42.18 (10 <sup>2</sup> )
Chitosaminic.....	-12.5	-24.37 (10 <sup>2</sup> )	Mannonic...	-14.25	-42.18 (10 <sup>2</sup> )
Dextro-xylohexos- aminic.....	+12.5	+24.37 (10 <sup>2</sup> )	Gulonic....	+14.25	+42.18 (10 <sup>2</sup> )
Levo-xylohexos- aminic.....	-12.5	-24.37 (10 <sup>2</sup> )	Idonic.....	-14.25	-42.18 (10 <sup>2</sup> )
Epichondrosaminic	+12.5	+24.37 (10 <sup>2</sup> )	Galaetonic ..	+ 8.25	+24.42 (10 <sup>2</sup> )
Chondrosaminic...	-12.5	-24.37 (10 <sup>2</sup> )	Talonic.....	- 8.25	-24.42 (10 <sup>2</sup> )
Dextro-ribohexos- aminic.....	+19.12	+37.28 (10 <sup>2</sup> )	Allonic.....	+20.8	+61.56 (10 <sup>2</sup> )
Levo-ribohexos- aminic.....	-19.12	-37.28 (10 <sup>2</sup> )	Altronic .....	-20.8	-61.56 (10 <sup>2</sup> )

Hudson was stimulated by our findings on the hexonic and hexosaminic acids and developed a more general conclusion, that the direction of the rotation of the carbon atom 2 determines the direction of the optical rotation of the acid. This is seen by mere comparison of our tables without resorting to calculation. Hudson arrived at his conclusion in a very ingenious way. He assumed that the superposition theory holds literally in every detail and he concluded that the magnitude and the direction of the rotation of any one of the four asymmetric carbon atoms

in hexonic acid can be calculated from four equations each representing the algebraic sum of the four asymmetric carbon atoms of one acid and of the value of its optical rotation in the following way.

1. *d*-Gluconic acid  $+ \alpha - \beta + \gamma + \delta = (+12.0) (286) = +34.3 (10^2)$
2. *d*-Gulonic "  $+ \alpha - \beta - \gamma + \delta = (+13.7) (286) = +39.2 (10^2)$
3. *d*-Idonic "  $- \alpha + \beta - \gamma + \delta = (-12.4) (286) = -35.5 (10^2)$
4. *d*-Galactonic "  $+ \alpha - \beta - \gamma + \delta = (+11.0) (286) = +31.5 (10^2)$

Solving these four equations, the following values are obtained:

$$\alpha = +37.3 (10^2); \beta = +3.9 (10^2); \gamma = +1.4 (10^2); \delta = -0.6 (10^2).$$

Weerman<sup>2</sup> later working on the amides of sugar acids, corroborated the conclusions of Levene<sup>3, 4, 5</sup>, and of Hudson. Hudson alone, and later with Komatsu<sup>6</sup> repeated the observations on a series of amides. They found the following values.

$$\alpha\text{-carbon} = +47.25 (10^2); \beta\text{-carbon} = -14.65 (10^2); \gamma\text{-carbon} = +0.95 (10^2); \\ \delta\text{-carbon} = -2.05 (10^2).$$

The authors then remark: "It will be noticed that the numerical values decrease as the carbon atom is further removed from the amide end. . . The alternation in the sign of the rotation of the successive carbon atoms is also noteworthy suggesting the alternation in positive and negative affinity that is often ascribed to the carbons in a chain." Inasmuch as we have found that the value of the rotation of carbon atom 2 ( $\alpha$ , according to Hudson's nomenclature) is not a constant for all acids, it was suggested that also the magnitudes of rotation of the other carbon atoms are influenced by the configuration of the adjacent carbon atoms. If that were so then the system adopted by Hudson for his calculations may be incorrect and may lead to erroneous conclusions.

We therefore calculated the magnitudes of rotation of each of the four carbon atoms in hexonic and 2-aminohexonic acids from several combinations of four equations. If the magnitudes

<sup>2</sup> Weerman, Dissertation, Amsterdam, 1916.

<sup>3</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 623.

<sup>4</sup> Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 367.

<sup>5</sup> Levene, P. A., and Clark, E. P., *J. Biol. Chem.*, 1921, xlv, 19.

<sup>6</sup> Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xli, 1141.

of rotation of each carbon atom were constant the same values should be obtained from all combinations.

It will be seen from the following table that the results obtained by Hudson and his coworkers are due to special conditions and that some combinations of four equations lead to values of rotation of the  $\alpha$ -carbon atom (Hudson's nomenclature) which are lower than some one other carbon atom.

In Combinations V, VI, and VII the values for the rotation of every carbon atom remain constant. These combinations are composed of members in which the  $\alpha$ -carbon atom is constant and equal to +12.5. On the other hand, in all combinations in which at least one member had the  $\alpha$ -carbon atom of the value 19.5 the values for the other carbon atoms were variable. It seemed therefore that the values for  $\beta$ -,  $\gamma$ -, and  $\delta$ -carbon atoms may possess constant and equal values when derived from the values of Part B of each acid, and not from that of the entire acid.

Calculations again showed that the values for  $\beta$ -,  $\gamma$ -, and  $\delta$ -carbon atoms remained equal and constant when derived from values of Part B of acids having the rotation of  $\alpha$ -carbon atom = 12.5, and not otherwise.

This observation naturally leads to the conclusions that the superposition theory holds only within certain limits.

In certain substances, as in hexonic acids, the vicinity of a certain group (carboxyl) accentuates the rotation of the  $\alpha$ -carbon atom to such an extent that the direction of its rotation determines the direction of the rotation of the entire molecule. The correctness of this conclusion with certainty can be demonstrated only on a comparison of the rotation of pairs of epimers.

It is, however, interesting to note that for the series of gluconic and mannonic, galactonic and talonic, gulonic and idonic, the superposition theory holds completely.

#### Hexosaminic acid.

I		
$[\alpha]_D$	$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +11.625$	$+22.65 (10^2)$
$\delta - \gamma - \beta + \alpha = +8.0$	$\beta = +2.125$	$+4.14 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = +0.125$	$+2.44 (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = -1.375$	$-26.82 (10^2)$

## II

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = + 6.75$	$+13.15 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\beta = - 5.75$	$-11.22 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = - 4.75$	$- 9.23 (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = - 9.25$	$-18.04 (10^2)$

## III

$\delta + \gamma + \beta - \alpha = -26.5$	$\alpha = +16.875$	$+32.95 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = - 1.375$	$- 2.68 (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = - 3.375$	$- 7.38 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = - 4.875$	$- 9.50 (10^2)$

## IV

$\delta + \gamma + \beta + \alpha = +12.5$	$\alpha = +19.5$	$+38.02 (10^2)$
$\delta + \gamma + \beta - \alpha = -26.5$	$\beta = + 7.0$	$+13.65 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\gamma = - 4.75$	$- 9.23 (10^2)$
$\delta - \gamma + \beta - \alpha = -17.0$	$\delta = - 9.25$	$-18.05 (10^2)$

## V

$\delta - \gamma - \beta + \alpha = + 8.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta + \gamma - \beta + \alpha = +10.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

## VI

$\delta - \gamma - \beta + \alpha = + 8.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

## VII

$\delta + \gamma - \beta + \alpha = +10.0$	$\alpha = +12.5$	$+24.38 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$\beta = + 3.0$	$+ 5.85 (10^2)$
$\delta - \gamma + \beta + \alpha = +14.0$	$\gamma = + 1.0$	$+19.50 (10^2)$
$\delta - \gamma - \beta + \alpha = + 8.0$	$\delta = - 0.5$	$- 0.98 (10^2)$

Phenylhydrazides of hexonic acids.

## VIII

	$[\alpha]_D$	$[\alpha]_D$	$[\alpha]_D$
$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = + 9.775$	$+27.95 (10^2)$	
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = + 8.375$	$+23.93 (10^2)$	
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = +10.625$	$+30.38 (10^2)$	
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = - 2.975$	$- 8.50 (10^2)$	

## IX

$\delta + \gamma + \beta - \alpha = -15.8$	$\alpha = +10.95$	$+31.32 (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\beta = -2.65$	$-7.58 (10^2)$
$\delta + \gamma - \beta - \alpha = -10.5$	$\gamma = -0.40$	$-1.15 (10^2)$
$\delta - \gamma + \beta - \alpha = -15.0$	$\delta = -1.80$	$+5.16 (10^2)$

## X

$\delta + \gamma + \beta + \alpha = +25.8$	$\alpha = +20.8$	$+59.48 (10^2)$
$\delta + \gamma + \beta - \alpha = -15.8$	$\beta = +7.15$	$+20.44 (10^2)$
$\delta - \gamma - \beta + \alpha = +12.2$	$\gamma = -0.35$	$-1.00 (10^2)$
$\delta - \gamma + \beta - \alpha = -15.1$	$\delta = -1.80$	$-5.14 (10^2)$

## XI

## Values of Part B.

	$[\alpha]_D$	$[M]_D$		$[\alpha]_D$	$[M]_D$
$\delta + \gamma + \beta + \alpha = +12.5$	$+24.37 (10^2)$		$\delta + \gamma + \beta = -6.75$	$-13.16 (10^2)$	
$\delta + \gamma + \beta - \alpha = -26.5$	$-51.67 (10^2)$				

## XII

$\delta - \gamma - \beta + \alpha = +8.0$	$+15.6 (10^2)$	$\delta - \gamma - \beta = -4.5$	$-8.58 (10^2)$
$\delta - \gamma - \beta - \alpha = -17.0$	$-33.15 (10^2)$		

## XIII

$\delta - \gamma + \beta + \alpha = +14.0$	$+27.3 (10^2)$	$\delta - \gamma + \beta = +1.5$	$+2.93 (10^2)$
$\delta - \gamma + \beta - \alpha = -11.0$	$-21.45 (10^2)$		

## XIV

$\delta + \gamma - \beta + \alpha = +10.0$	$+19.5 (10^2)$	$\delta + \gamma - \beta = -2.5$	$-4.88 (10^2)$
$\delta + \gamma - \beta - \alpha = -15.0$	$-29.25 (10^2)$		

Solving for XII, XIII, and XIV, the values obtained are  $\beta = +3.0$ ,  $\gamma = +1.0$ , and  $\delta = -0.5$ .

Solving for XI, XII, and XIII, the values obtained are  $\beta = +3.0$ ,  $\gamma = -4.125$ , and  $\delta = -5.625$ .

# THE PREPARATION AND STANDARDIZATION OF COLLODION MEMBRANES.

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The use of collodion membranes in the study of diffusion phenomena was begun by Fick (1855); the closed collodion sac was devised 5 years later by Schumacher (1860). Metchnikoff, Roux, and Taurelli-Salimbeni (1896), in their researches on the vibrio of Asiatic cholera introduced these sacs into biological science. Because of their ease of preparation, availability, thinness, and ready permeability to ordinary crystalloids, they have largely replaced the older parchment paper membranes in the study of dialysis and diffusion. For a complete bibliography and literature review of this development of their use, the reader is referred to the paper of Bigelow and Gemberling (1907). Mention should be made, however, of the work of Gorsline (1903), who showed that crystalloids were not the only substances that could diffuse through a collodion membrane, for at 35°C., peptone, albumose, albumin, starch, dextrin, and certain enzymes all dialyzed through in less than 24 hours in recognizable quantities.

The first successful attempt to produce a graded series of membranes of increasing permeability was made by Bechhold (1907, 1908). By impregnating filter papers with varying percentage strengths of gelatin or glacial acetic acid collodion, he produced membranes whose permeability varied inversely with the concentration of the impregnating solution. These membranes were used as filters. With the use of pressure, often many atmospheres, substances like hemoglobin, albumin, and various inorganic colloids were filtered through. A mechanical stirring device was employed to prevent the precipitation of the colloid on the surface of the membrane; such precipitated colloid

otherwise forming a very impervious film. The higher the pressure the more prone is this superimposed film to form. In a former investigation, De Kruif and the author (1919) were unable to prevent the formation of this film in the filtration of anaphylatoxin, the membranes becoming rapidly less permeable as filtration proceeded.

Another method of increasing and regulating the permeability of collodion membranes is that of Schoep (1911). Increased permeability is secured by adding 2 to 10 per cent of glycerol to an alcohol-ether solution of Schering's celloidin; 4 per cent of castor oil is added to give the membranes elasticity. A wide range of permeability is secured by varying the amount of glycerol added. These membranes are far more permeable than those of Bechhold; very low pressures are sufficient for the filtration of substances like albumin or hemoglobin. There is little tendency to form the objectionable colloidal film on the filtering surface. De Kruif and Eggerth (1919) used them with success in filtering the greater part of the serum proteins from anaphylatoxin.

Brown (1915) developed another method of varying permeability. Collodion sacs are air-dried and then immersed in mixtures of ethyl alcohol and water; the greater the percentage of alcohol, the more they swell, and the more permeable is the resulting membrane. This method gives a valuable series, but even the most permeable members of it (those treated with 90 to 96 per cent alcohol) do not allow much diffusion of substances such as Congo red or hemoglobin; dialysis for 2 to 7 days allows only small quantities to pass through. The Brown series, excellent in its scope, needs to be extended in the direction of greater permeability.

The Schoep membranes seem to answer that need. But the Schoep membranes are made from Schering's celloidin, which cannot be purchased in the United States now. Several brands of American pyroxylin were tried in the Schoep formulas with poor success. If little glycerol was used, the membranes were very impermeable; if the amount of glycerol was increased, they were so fragile as to be useless. Considerable improvement was effected by substituting 10 per cent of acetic or lactic acid for the castor oil used by Schoep; a stronger membrane with a

considerable range of permeability was obtained. While attempting to adapt the Schoep formulas to the available pyroxylin, it was observed that merely altering the proportion of alcohol to ether in the solvent mixture sufficed to alter the permeability of the resulting membranes through a wide and easily controllable range. These membranes have the advantage of being free from castor oil, they are transparent, and stronger than those of Schoep.

A similar observation has been made by Malfitano, who states (1908) that the higher the proportion of alcohol in the solvent, the more permeable the membrane, and in 1910 he stated that Michel and Lazarus in his laboratory had demonstrated that alcohol-rich solutions give more permeable membranes than those that are ether-rich. No further details are given.

The pyroxylin used in this investigation is that manufactured by Du Pont De Nemours and Company under the name of parlodion. All of the solutions referred to in this paper contained 6 gm. of the dry collodion in 100 gm. of alcohol-ether solvent. The parts of alcohol and ether were all taken by weight. To designate the different solutions and membranes, the following nomenclature was adopted: a solution is named according to the parts by weight of alcohol it contains; one containing 50 parts of alcohol and 50 of ether is a "50 alcohol solution;" a membrane made from this solution is designated a "50 alcohol membrane."

The ethyl alcohol first used in making up the solvent was distilled over anhydrous copper sulfate; later in the work it was distilled over calcium oxide and redistilled over metallic sodium. The ether was distilled over sodium.

Alcohol-ether mixtures containing from 20 to 80 parts of alcohol by weight readily dissolve 6 per cent of parlodion; but to obtain solutions containing 10, 15, 85, and 90 parts of alcohol, it is necessary first to make a thick solution of the collodion in a portion of the solvents and later add the remainder of the alcohol or ether, as the case may be.

On adding the last part of the ether to the 10 alcohol solution of collodion, the solution has on two occasions set to a gel, which could be redissolved by adding another per cent of alcohol. On two other occasions, a white flocculent precipitate formed, which failed to redissolve. This was allowed to settle out, and the supernatant solution was used in making the membranes.



The different collodion solutions differ considerably in their viscosity, as can be seen by referring to Table I. Viscosities were determined rather roughly by noting the time in seconds required for the solutions to flow out of the same 10 cc. pipette. The 20 alcohol solution was found to be the least viscous, viscosity increasing regularly in both directions in the series.

The changes in viscosity of these solutions after a small amount of water has been added are noteworthy. The alcohol-rich

TABLE I.  
*Relative Viscosities at 20°C.*

Solution.	Anhydrous.	5 per cent of water added.	Remarks.
10 alcohol.	35	18	Transparent emulsion.
15 "	26		
20 "	20	15	Granular precipitate. Redissolves readily.
30 "	23		
40 "	29	26	Granular precipitate. Redissolves readily.
50 "	34		
60 "	40	41	Gelatinous precipitate. Redissolves with difficulty.
70 "	45		
80 "	53	85*	Sets to gel in 24 hours.
85 "	64		
90 "	85	—*	Sets to gel.

\* 2 per cent of water added.

solutions are made more viscous (only 2 per cent of moisture setting the 90 alcohol solution to a firm gel) while the ether-rich solutions are made more fluid by the same agent. Several batches of 90 alcohol solutions, made up with supposedly anhydrous solvents, slowly increased in viscosity, and after several days, set to a semisolid gel; not until the last traces of water were removed from the alcohol by redistillation over sodium was a 90 alcohol solution obtained that could be kept for weeks without marked change in viscosity.

*Method of Making the Sacs.*

The collodion sacs were made by the method devised by Novy,<sup>1</sup> with a few slight modifications. A glass tube, melted down at one end to leave a small hole, is the mold. A small fragment of cigarette paper is slightly moistened and placed over the hole, where it dries quickly; a layer of collodion is painted over the paper and the end of the tube; this is allowed to dry for 20 to 30 seconds. A few cubic centimeters of collodion solution are poured into a test-tube; this is held nearly horizontal while the end of the mold tube is immersed in the collodion. The mold tube is rotated slowly, and slowly withdrawn; then it, with its covering of collodion, is thrust horizontally into a large test-tube that lies on the table before the operator, the mold tube being rotated rapidly all the time. In these experiments the membranes were always dried for 1 minute, and then immersed in water. Rotating the membrane within the large test-tube in the manner described makes the drying slower and more uniform, and cuts off air currents. It was found to be good practice never to return any unused collodion to the stock bottle.

By filling the mold tube with water and applying air pressure to the open end with the mouth, it is usually not difficult to force water between the glass and the membrane, and so easily remove the latter. The tube and the membrane should be immersed during this process. Sometimes the membranes adhere to the glass at the edge of the hole, and no amount of gentle manipulation with the fingers can free them. Increased pressure finally breaks through, but often tears the membrane. If a short rubber tube (not too thin-walled) is slipped over the end of the mold tube and filled with water, and a small cork stopper is pushed slowly down the rubber tube, the membrane can be "started" with ease and without tearing, this expedient allowing the application of a great deal of force with a minimum of displacement. By pinching on the rubber tube as the cork is withdrawn, too much negative pressure is avoided. After loosening the membrane in this way, it can be easily removed by blowing with the mouth. The ether-rich membranes (the

<sup>1</sup>A description of this method is given in the paper by Bigelow and Gemberling (1907).

20, 30, and 40 alcohol solutions), adhere very tightly to the glass and the percentage of spoiled membranes is very high unless the rubber tube and cork are used. The 20 and 30 alcohol membranes require in addition a little manipulation with the fingers at the very end to free them.

The 10 and 15 alcohol membranes adhere so tightly to the glass that they cannot be made by this method at all, nor can they be made on the inside of a test-tube. A solid glass rod was ground down so that it tapered from a diameter of 11.0 mm. at a point 8 cm. from the end to 10.6 mm. at the end itself. The end of the rod is dipped into the collodion solution to a depth of about 1 cm.; this coat is allowed to dry for 15 to 20 seconds, and then the entire membrane is made as described above. Two coats are necessary at the extreme end, otherwise this portion is so thin that it is certain to tear. After immersing in water, the membrane is removed by peeling it down from the top. For other membranes of the series, this method does not work so well as the one first described.

#### *Method of Conducting Diffusion Experiments.*

The sacs, usually prepared on the preceding day, were each attached by means of a rubber band to a glass tube about 8 cm. long; this glass tube passed through a cork which had a longitudinal groove cut in the side. The corks fitted into the necks of test-tubes of  $\frac{1}{2}$  inch diameter. Sacs made by the first method described had a diameter of 8.3 mm. in all experiments, 3 cc. of test substance were placed in each sac, which filled it to a depth of 6 cm. The volume of the dialysate was always 10 cc., this kept constant throughout the experiment. The sacs were always immersed to a depth of 6 cm. The membranes made by the second method were larger, with an average diameter of 10.8 mm. 5 cc. of test substance were used, and they were immersed to a depth of 8 cm. All of the membranes used in the diffusion of NaCl,  $\text{KH}_2\text{PO}_4$ , saccharose, and indigo carmine (Fig. 3) were made by the second method.

In the diffusion experiments performed, four or five and sometimes more sacs made from the same solution were tested each time. Thus the experiment plotted in Fig. 1 required thirty sacs, five sacs of each of six grades of permeability.

Wherever possible, quantitative determinations of the amount of test substance that had passed through the membranes were made at different time intervals and the results plotted as in Figs. 1 and 2.

*Test Substances and Method of Their Estimation in the Dialysate.*

*Two Per Cent Aqueous Sodium Chloride.*—Estimated by titration with a standard silver nitrate solution, using potassium dichromate as an indicator.

*0.2 M Potassium Dihydrogen Phosphate.*—Estimated by titration with 0.1 N sodium hydroxide using phenolphthalein as an indicator and titrating to a full red color.

*Saccharose, 20 Per Cent Solution.*—Estimated with the polarimeter.

*Raffinose, 8 Per Cent Solution.*—Estimated with the polarimeter.

*Indigo Carmine (Sodium Sulfindigotate), 0.2 Per Cent Aqueous Solution.*—Estimated with the colorimeter.

*Safranine, 1 Per Cent Aqueous Solution.*—Estimated with the colorimeter.

*Primary Proteose, 1 Per Cent Aqueous Solution at pH = 7.0.*—This was prepared from Difco peptone by precipitating with  $(\text{NH}_4)_2\text{SO}_4$ , and purified by reprecipitating three times with  $(\text{NH}_4)_2\text{SO}_4$  to half saturation. The proteose was estimated by adding 6 cc. of saturated  $(\text{NH}_4)_2\text{SO}_4$  to 4 cc. of the dialysate and determining the precipitate nephelometrically.

*Dialyzed Serum Protein.*—No effort was made to separate the albumin from the pseudoglobulin, but the euglobulin was removed by centrifugation. To 10 cc. of sheep serum 1.8 cc. of 0.2 N HCl were added; this brings the serum approximately to the optimum reaction for the flocculation of euglobulin. This was then dialyzed in a 40 alcohol membrane against distilled water. The volume was then made up to three times the original volume of the serum. In one experiment, the protein in the dialysate was precipitated with sulfosalicylic acid and determined nephelometrically; in another, the nitrogen was determined by the micro-Kjeldahl method of Folin and Wu (1919), and the protein computed.

*Oxyhemoglobin*.—Sheep cells were washed three times with isotonic salt solution. An equal volume of distilled water was added to the packed cells and cooled; then a half volume of cold ether was added and shaken. After standing in the ice box over night, the lower layer was drawn off and diluted to a volume ten times as great as that of the original packed cells. It was estimated with the colorimeter.

*Carboxyhemoglobin*.—Carbon monoxide, prepared from oxalic acid and sulfuric acid, was bubbled through a solution of oxyhemoglobin prepared as above.

*Methemoglobin*.—To the concentrated oxyhemoglobin solution was added a half volume of 10 per cent potassium ferricyanide; this was now dialyzed in a large 40 alcohol sac until the dialysate was free from ferricyanide. It was estimated colorimetrically.

*Congo Red (Grubler's)*.—A 1 per cent aqueous solution was used. This has a pH of about 8.6. It was estimated colorimetrically.

In the diffusion experiments the proteose, serum protein, and hemoglobin solutions were dialyzed against a buffered phosphate solution set at pH 7.0, made by adding 29.6 cc. of 0.2 N NaOH to 50 cc. of 0.2 M  $\text{KH}_2\text{PO}_4$  and diluting to 300 cc. The other test substances were dialyzed against distilled water. An effort was made to stabilize the reaction of the Congo red solution by using a buffered dialysate, but the presence of electrolytes greatly reduces the solubility of Congo red, so this was abandoned.

Fig. 1 shows the passage of Congo red through membranes of six grades of permeability. The 85 alcohol membranes allow very rapid diffusion of this typical "non-dialyzable colloid," while the 40 alcohol membranes allow only traces to pass through in the first 2 or 3 hours. The 30 alcohol membranes, not shown on the figure, allow no diffusion in 24 hours. In 5 hours, the 85 alcohol membranes have passed twelve times as much Congo red as the 50 alcohol ones.

By referring to Table II it will be seen that the thickness of these membranes increases towards the alcohol-rich end of the series. The higher alcohol membranes cannot be made any thinner without impairing their strength; any attempt to secure uniformity of thickness in the entire series would involve thickening the ether-rich membranes to an unnecessary and undesirable extent. It is obvious that if the membranes had

all been of the same thickness, the differences between the curves shown would, if anything, be greater; certainly not less, since the alcohol-rich membranes are the thickest and at the same time the most permeable.

A 1 per cent solution of Congo red exerts an osmotic pressure which causes an increase in the volume of the dialysee. This increase in volume is greatest with the least permeable mem-

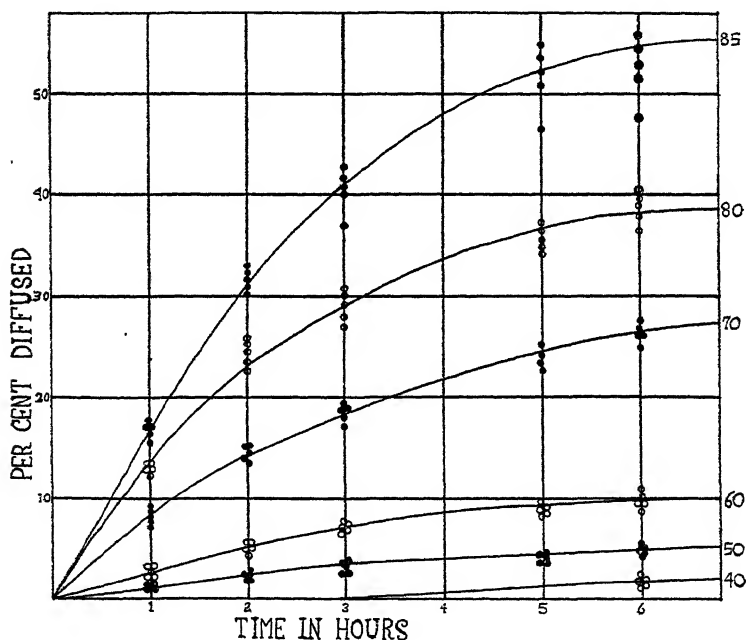


Fig. 1. Diffusion of 1 per cent aqueous Congo red at 35°C. through membranes of six grades of permeability. The numbers at the margin are the alcohol numbers of the membranes.

branes, and becomes less as permeability increases. Thus, the 40 alcohol membranes raised a column averaging 18.0 cm. in 8 hours; the 85 alcohol membranes raised a maximum column of 3.0 cm. in 3 hours, after which it began to fall. With the aid of a ruler, it was possible to place the thirty membranes used in this experiment in their correct order of permeability. Other test substances, such as hemoglobin and serum protein,

TABLE II.

Membrane.	Area = A. sq. cm.	Empty space = r. mm.	Thickness (measured). mm.	Thickness (calculated) = L. mm.	Time = T. min.	Amount filtered = F. cc	$\frac{F L}{A r T} = Q L.$	$\frac{1}{V/Q L}$	Wet weight. gm.	Dry weight gm.	Ratio.
10 alcohol.	25.1	0.54	0.017	0.014	380	1.2	0.00033	0.13	0.0380	0.0220	1.73
	22.9	0.60	0.015	0.011	380	0.8	0.00017	0.11	0.0290	0.0151	1.92
	24.8	0.62	0.015	0.011	380	0.6	0.00011	0.10	0.0290	0.0148	1.95
15 "	23.7	0.71	0.011	0.010	200	1.5	0.00045	0.15	0.0250	0.0099	2.5
	23.7	0.63	0.012	0.012	200	1.1	0.00039	0.14	0.0350	0.0164	2.1
	23.7	0.62	0.011	0.011	200	1.0	0.00033	0.13	0.0330	0.0165	2.0
20 "	18.2	0.71	0.015	0.011	187	4.5	0.00210	0.21	0.0200	0.0080	2.5
	21.9	0.73	0.011	0.010	215	4.6	0.00130	0.19	0.0190	0.0070	2.7
	21.4	0.71	0.011	0.009	187	2.2	0.00075	0.17	0.0170	0.0067	2.5
30 "	23.5	0.81	0.015	0.013	54	7.1	0.00890	0.31	0.0240	0.0072	3.3
	23.0	0.78	0.016	0.013	90	7.2	0.00580	0.28	0.0230	0.0070	3.3
	19.2	0.77	0.014	0.011	102	6.7	0.00490	0.26	0.0200	0.0064	3.1
40 "	19.8	0.82	0.020	0.021	34	6.7	0.02500	0.40	0.0370	0.0095	3.9
	22.2	0.83	0.017	0.019	25	6.0	0.02000	0.37	0.0340	0.0083	4.1
	23.5	0.83	0.015	0.015	34	7.1	0.01600	0.36	0.0270	0.0066	4.1
50 "	24.0	0.84	0.035	0.034	35	5.5	0.02800	0.41	0.0560	0.0115	5.0
	22.7	0.84	0.025	0.028	35	6.0	0.02500	0.40	0.0480	0.0095	5.0
	23.0	0.84	0.035	0.032	35	5.5	0.02500	0.40	0.0560	0.0114	5.0
60 "	23.5	0.89	0.040	0.043	27	6.5	0.04900	0.47	0.0730	0.0116	6.3
	25.6	0.89	0.030	0.035	24	6.3	0.03500	0.43	0.0600	0.0099	6.1
	25.6	0.90	0.035	0.043	24	5.2	0.03300	0.43	0.0730	0.0106	6.9
70 "	27.2	0.92	0.050	0.050	17	6.8	0.08000	0.53	0.0840	0.0104	8.0
	26.7	0.90	0.040	0.038	17	6.8	0.06600	0.51	0.0650	0.0095	6.9
	23.5	0.91	0.045	0.040	17	4.6	0.05700	0.49	0.0680	0.0095	7.2
80 "	24.0	0.93	0.065	0.074	18	5.6	0.10300	0.57	0.1230	0.0133	9.2
	23.2	0.92	0.060	0.060	17	5.5	0.09100	0.55	0.1000	0.0120	8.3
	22.4	0.92	0.060	0.051	18	5.6	0.07700	0.53	0.0860	0.0107	8.0
85 "	23.0	0.93	0.055	0.070	12	6.4	0.17000	0.65	0.1170	0.0124	9.4
	25.6	0.93	0.052	0.063	12	7.0	0.16000	0.63	0.1050	0.0117	9.0
	21.4	0.93	0.050	0.055	12	6.0	0.14000	0.61	0.0920	0.0102	9.0
90 "	22.7	0.93		0.090	12	5.2	0.18500	0.66	0.1500	0.0165	9.1
	24.8	0.93		0.083	17	7.3	0.15000	0.63	0.1380	0.0147	9.3
	24.0	0.93	0.080	0.080	17	6.0	0.12600	0.60	0.1340	0.0146	9.3

which were dialyzed against a buffered phosphate solution, showed the same phenomenon, though here the columns raised did not exceed 1 to 2 cm. during the time of the experiment.

Fig. 2 shows in detail the diffusion of methemoglobin through the same six grades of membranes. It will be observed that diffusion through the 40, 50, 60, and 70 alcohol membranes is more rapid than with Congo red; for the 85 alcohol membranes,

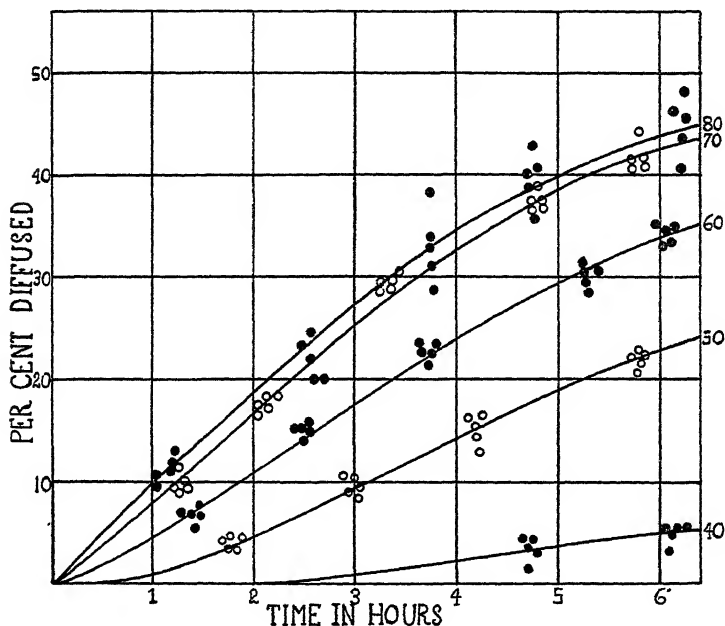


FIG 2. Diffusion of methemoglobin at 35°C. The numbers at the margin are the alcohol numbers of the membranes. The 85 alcohol series is omitted as the points have the same distribution as for the 80 alcohol series.

it is slower. Also, there is no essential difference in the diffusion of this substance through the 70, 80, and 85 membranes. It is probable that the methemoglobin molecule is smaller than that of Congo red, hence it passes more easily through the less permeable membranes. Further increase in permeability beyond the 70 alcohol membrane can no longer hasten the diffusion, which is limited by the diffusion constant of methemoglobin in water.



Congo red, with a larger aggregate, is more retarded by the lower membranes of the series; but being highly ionized relative to the methemoglobin, its diffusion constant in water is greater and it passes the 85 alcohol membranes more rapidly than the latter substance.

The curves for oxyhemoglobin and carboxyhemoglobin are not given, as they so closely resemble those of methemoglobin.

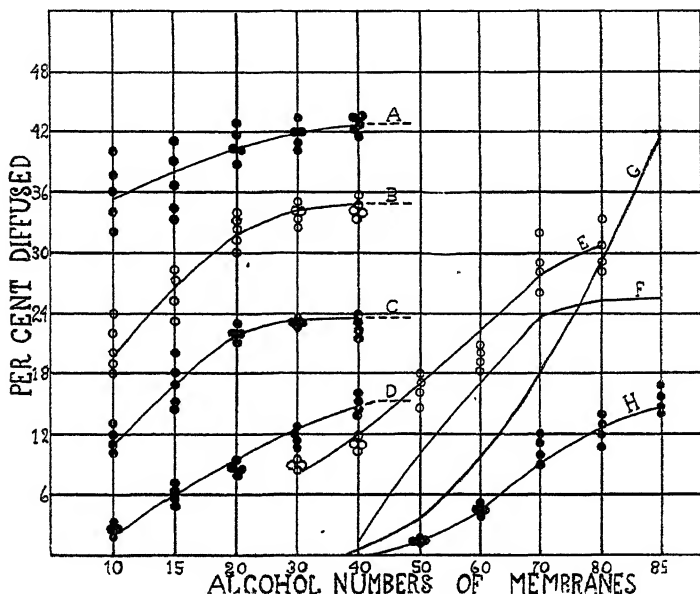


FIG. 3. A, sodium chloride, 10 minutes at 20°C. B, potassium dihydrogen phosphate, 10 minutes at 20°C. C, saccharose, 30 minutes at 20°C. D, indigo carmine, 20 minutes at 20°C. E, primary proteose, 2½ hours at 20°C. F, methemoglobin, 3 hours at 35°C. G, Congo red, 3 hours at 35°C. H, dialyzed serum, 3 hours at 35°C.

The behavior of indigo carmine and safranin was also studied in detail. Indigo carmine diffuses very slowly through the 10 alcohol membranes; the speed of diffusion increases with the alcohol numbers up to the 40 alcohol membranes, after which there is no further appreciable increase. The behavior of this substance is given in abbreviated form in Fig. 3. Safranin is somewhat less diffusible than indigo carmine; otherwise their curves are very similar.

Test substances, not colored, were tested for one time interval only. The behavior of these substances is shown in Fig. 3. Here the alcohol numbers of the membranes are plotted against the percentage of test substance diffused; the times and temperatures are indicated. Congo red and methemoglobin are included for comparison. The points on these two curves are omitted for the sake of simplifying the figure, as they may be found in Figs. 1 and 2. The curves of oxyhemoglobin and carboxyhemoglobin, safranin, and raffinose are also omitted for the same reason. With all the test substances, it is apparent that the permeability of the membranes increases with their alcohol numbers.

It is not altogether certain that the rise in the sodium chloride curve represents a real rise in the permeability of the membrane series to this test substance. If we consider a membrane as made up of a solid meshwork containing water-filled pores or spaces, then the true diffusing area is not the area of the membrane, but the sum of the areas of the pores. The proportion of water-filled spaces is not the same throughout the membrane series, as may be seen by referring to Column 3 of Table II. The 20 alcohol membranes have, on this theory, one-sixth more water-filled space than the 10 alcohol membranes of the same area. This is sufficient to account for the rise of the sodium chloride curve. This line of reasoning will not, however, explain away the rise in the curves of the other test substances studied as over 50 per cent more of phosphate, and 100 per cent more of saccharose diffused through the 20 alcohol membrane than through the 10, in the same length of time. The 70 alcohol membranes have about one-twelfth more water-filled space than the 50 alcohol membranes, yet twenty times as much serum protein diffused through them in the same length of time.

Fig. 4 shows the effect of different temperatures on the rate of diffusion of methemoglobin through 70 alcohol membranes. At the lower temperatures it will be noticed that the rise in the curve is preceded by an appreciable flat portion, representing a period during which little or no test substance diffuses through. The membrane, apparently, must be "saturated" with the substance before any of it can appear in the dialysate. If a methemoglobin solution is filtered under low pressure, through a 60.

or 70 alcohol membrane, the first few cubic centimeters are colorless; only after the membrane is saturated with the test substance will any of it come through. If a very dilute solution, such as 1 to 1,000 Congo red, is used in diffusion experiments, little or none may appear in the dialysate even when the membrane is very permeable to that substance. To obtain consistent results with Congo red, it was found necessary to use a more

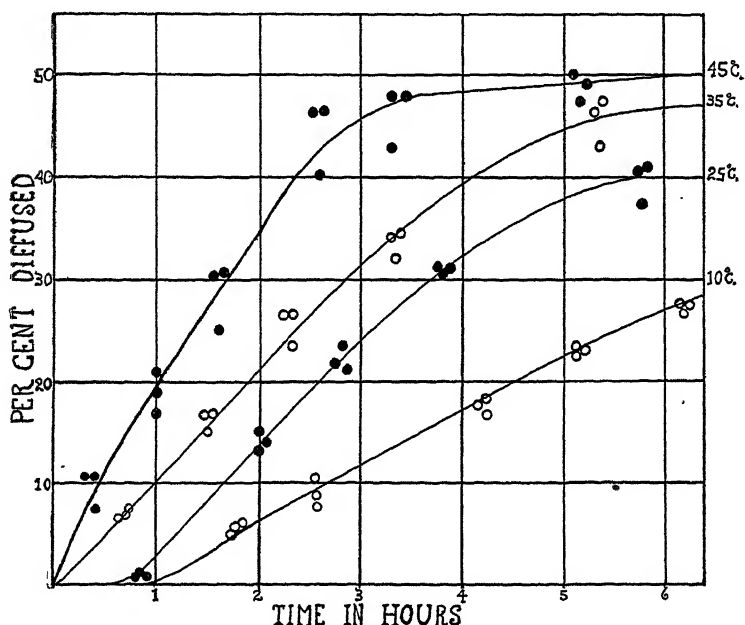


FIG. 4. Diffusion of methemoglobin through 70 alcohol membranes at different temperatures.

concentrated solution, so that the amount taken up by the membrane is small in comparison to the total amount.

The velocity of the filtration of water through membranes was used by Bechhold (1908), not only for demonstrating differences of permeability but also for calculating the diameters of the pores. This method is based upon the application to membranes of the law of Poiseuille for the passage of fluid through a capillary. The membrane is considered as a number of capillaries whose length is the thickness of the membrane.

This law states that  $Q = k \frac{PD^4}{L}$ ; where  $Q$  is the quantity of fluid passed through unit area in unit time,  $P$  the pressure,  $D$  the diameter of the capillary (pore),  $L$  the length of the capillary (thickness of membrane), and  $k$  a constant depending on the nature of the fluid, the temperature, and the units chosen. From this equation, it follows that  $D = \sqrt[4]{\frac{QL}{kp}}$ . Hence, with

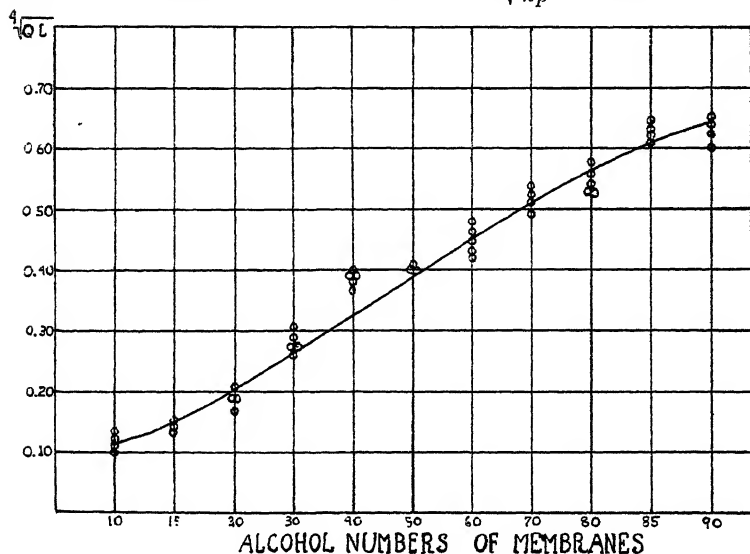


FIG. 5. Values of  $\sqrt[4]{QL}$  for the membrane series. Pressure, 60 mm. of Hg; temperature, 20°C.

constant pressure and temperature,  $D$  will vary as  $\sqrt[4]{QL}$ . The value of  $QL$  can be readily calculated from the experimental data. This has been done in Table II. Only three membranes of each series are given in this table, selected to show maximum, mean, and minimum values of  $QL$ . The values of  $\sqrt[4]{QL}$  are plotted in Fig. 5. No attempt was made to determine the value of the constant  $k$ , without which  $D$  cannot be computed.

#### *Method of Conducting Water Filtration Experiments.*

Bulbs of about 10 cc. capacity were blown from 8 mm. tubing; the two ends were cut down to 2 cm. in length. Each membrane

was attached to one of the ends of a bulb with a strong rubber band; the joint was dried with a towel and painted over with a thick 30 alcohol solution of collodion. When dry, the joint was tested for air-tightness. The sac was emptied and 12 cc. of distilled water were measured into it. A rubber tube over the other end of the bulb, led to a pressure tank and manometer. The filled sac was completely immersed in a test-tube of water, which stood in a constant temperature bath. A pressure of 60 mm. of Hg and a temperature of 20°C. were used in all experiments. Filtration was continued until the bulb, but not the sac, was empty. After filtration, the sac was emptied and tested for leaks; the volume of the residual water was measured.

The thickness of the membranes was measured with a micrometer, taking the mean of several readings at different places. The thickened bottom of the sac was removed; a piece 6 cm. long was cut off, rapidly blotted between filter papers, and weighed in a weighing bottle. This gave the wet weight. Each membrane was then air-dried and weighed again. The specific gravity of dry collodion was found to be 1.608 at 20°C.

The volume in cubic centimeters occupied by the membrane is then equal to

$$\text{Wet weight} - \text{dry weight} + \frac{\text{dry weight}}{1.608}$$

all weights being expressed in grams. Dividing this value by the area of the membrane gives its thickness (Column 5, Table II). As the thickness obtained in this manner gave more consistent results than those obtained by measuring with the micrometer, this was used in calculating the value of  $QL$ . In making this calculation, the unit for  $L$  was taken as 0.01 mm.

The ratio of water-filled space to total volume was calculated as follows:

$$r = \frac{\text{wet weight} - \text{dry weight}}{\text{volume}}$$

Walpole (1915) and Brown (1915, 1917) showed that the ratio of the dry to the wet weight of a membrane was an index of its permeability. Their conclusion is fully supported by this investigation, as may be seen in the last column of Table II.

In the course of this investigation, a number of other substances were added to the alcohol-ether solvents, in the hope of improving the series. The most promising were certain of the organic acids. If acetic acid, for instance, is added to the solvent, it becomes much easier to make the membrane; instead of shrinking to the glass on immersing in water, the collodion film loosens from it readily and slips off with ease. 10 to 20 per cent of acetic acid seems also to give added durability and elasticity to the resulting membrane, and it increases the amount of non-solvent, such as glycerol or water, that may be added to the solution. The addition of this amount of acetic acid makes the membranes less permeable (only the 50 and 70 alcohol membranes were tried). Oxalic and citric acids and phenol made 70 alcohol membranes slightly more permeable when added in 5 per cent amounts. Lactic acid, however, added to the solutions in amounts varying from 10 to 30 per cent, greatly increases the permeability of the resulting membranes. Glycerol and water both increase permeability, though neither are as effective as lactic acid.

Since the work of Metchnikoff, collodion sacs have been extensively used in bacteriology and serology. For this work, it is desirable to sterilize the membranes by heat. Heating is accompanied by two undesirable changes, marked shrinkage and marked decrease in permeability. This matter has recently been investigated by Gates (1921), who gels his membranes in 95 per cent alcohol before immersing them in water; on heat sterilization, his membranes shrink about 33 per cent in volume and are still readily permeable to simple salts and glucose, though quite impermeable to protein and hemoglobin. It would seem desirable to prepare membranes that would allow the passage of proteins after heat sterilization; with this in view, the following experiments were conducted:

Sacs were prepared from the 80 alcohol solution. Some were immersed in 95 per cent alcohol, after the method of Gates. Others were made from the 80 alcohol solution plus 10 per cent of lactic acid, and immersed directly into water and washed free from acid. When autoclaved at 20 pounds pressure for 30 minutes, both sets of membranes shrank over 50 per cent in volume; when sterilized by three steamings in the Arnold

sterilizer, the shrinkage was very nearly 33 per cent for both sets of membranes. When tested with methemoglobin solution at 35°C., both sets of membranes were found to transmit it readily, the lactic acid membranes being more permeable. The latter had the permeability of unheated 60 alcohol membranes. Sacs made by both methods are serviceable and strong; they will stand over 25 cm. Hg of pressure without bursting. *Staphylococcus aureus* was grown in such sterile membranes for 2 months without contaminating the surrounding broth. *Bacillus influenzae* was grown in pure culture in plain broth without hemoglobin by growing it on the inside of a sterilized sac, with a living culture of staphylococcus, streptococcus, or pneumococcus growing on the outside; controls without the symbiotic organisms showed no growth of *Bacillus influenzae*. By this means it is possible to study the symbiosis of organisms while keeping each of them in pure culture.

The value of a method depends, among other things, on whether the results with it can regularly be reproduced. Most of the experiments described above were repeated many times, in whole or in part; fresh collodion solutions were made up several times from different lots of materials. Results obtained with membranes in the series between the alcohol numbers of 20 and 85 could be reproduced without any trouble. Such variations as occurred between different batches are believed to have been due to the presence of small amounts of water in solution. With the extremes of the series, more difficulty was encountered. The 90 alcohol membranes gave such irregular results that they have not been included in the series; they were several times found to be less permeable than the 85 or even the 80 alcohol membranes. This may in part have been due to their great variations in thickness, as the viscosity of the 90 alcohol solutions was found to be very variable. By referring to Table I, it will be seen that very small quantities of moisture suffice to cause great changes in viscosity. Variations in permeability were also found in the 10 and 15 alcohol membranes of different batches. One batch gave an almost horizontal line for the diffusion of sodium chloride and potassium dihydrogen phosphate, when plotted out as in Fig. 3. Three other batches gave more constant results.

## SUMMARY.

A simple method of preparing a graded series of collodion membranes of a wide range of permeability is presented, with quantitative data on the diffusion of various test substances through the different grades of the series.

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# THE DIRECT QUANTITATIVE DETERMINATION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IN SMALL AMOUNTS OF BLOOD.

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The concentration of sodium, potassium, calcium, and magnesium in the blood of animals has been determined by Bunge (1), Abderhalden (2), and more recently by Greenwald (3). These investigators used from 25 to 100 cc. of blood for their determinations. Such quantities of blood cannot conveniently be used in studies with patients, particularly children. We have therefore devised a method by means of which the concentration of all these elements may be quantitatively determined on 7 cc. of blood.

## *Principle of the Method.*

Deproteinization is carried out by means of the trichloroacetic acid method recommended by Greenwald (4). The determinations of the individual elements, except that of magnesium, are then made directly on separate aliquots of the deproteinized fluid by modifications of methods previously described by us for serum (5, 6, 7). The inorganic or acid-soluble phosphorus may also be determined by any of the well known micro methods (8) on a portion of the supernatant fluid corresponding to 0.5 or 1.00 cc. of blood.

## *Methods.*

*Collection of Material and Deproteinization.*—25 cc. of distilled water are placed in a 50 cc. volumetric flask which is then weighed. From 7 to 8½ cc. of blood are obtained by means of a 10 cc. graduated syringe and slowly added to the water in the flask. The flask should be continuously rotated during this procedure which

completely hemolyzes the blood. The flask and contents are again weighed and the exact amount of blood added thereby determined. 1 or 2 drops of octyl alcohol are added followed by 12 to 13 cc. of 12 per cent trichloroacetic acid which are added slowly while the flask is rotated. The contents are thoroughly mixed and allowed to stand 10 minutes. Water is added to 50 cc., the contents are again mixed, transferred to a large centrifuged tube and centrifuged for 5 to 10 minutes at about 1,000 revolutions per minute. The supernatant fluid is poured off and an aliquot (generally 35 cc.) is placed in a beaker and evaporated. The presence of a few particles of the precipitate which sometimes float on the surface of the supernatant fluid does not interfere with the subsequent determinations. If the particles are very numerous, the fluid may be allowed to stand in the ice chest for a few hours. They will then have settled to the bottom. The supernatant fluid may be kept at this stage for at least 2 weeks before completing the determination. After the aliquot has been evaporated to dryness the residue is dissolved in 0.1 N HCl and is transferred to a volumetric flask and the volume made up to 10 cc. This fluid has the appearance of serum. Should it be cloudy it may be centrifuged for a few minutes when a clear, straw-colored supernatant liquid will be obtained. The sodium, potassium, and calcium determinations are done directly on this material while the magnesium determination is done on the first supernatant fluid obtained from the calcium determination.

#### *Sodium Method.*

4 cc. of the material prepared as outlined above are placed in a platinum dish and evaporated to about 2 cc. A drop of phenolsulfonephthalein is added and the contents are made just alkaline with 10 per cent KOH (generally about 10 to 12 drops will suffice). 10 cc. of the potassium pyroantimonate reagent are added followed by 3 cc. of 95 per cent alcohol. The alcohol should be added drop by drop and the specimen stirred with a rubber-tipped rod. After standing 30 minutes the precipitate is transferred to a weighed Gooch crucible and washed with 5 to 10 cc. of 30 per cent alcohol. The crucible is dried at 110° C. for 1 hour,<sup>1</sup> cooled in a desiccator for 30 minutes, and weighed.

<sup>1</sup> The temperature should be gradually raised to 110°C.

The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

The method of preparation of the potassium pyroantimonate reagent has been fully described in a former paper on the determination of sodium in serum (5). The details of the method of preparation of the Gooch crucibles and the precautions to be observed during the addition of the alcohol and the filtration, and also the care of the platinum are fully outlined in the same paper.

For the determination of sodium in solutions of blood ash we used the same procedure as described for the determination of this element in solutions of the ash of urine and stools (9).

#### *Potassium Method.*

0.2 cc. of the material prepared as previously outlined is placed in a graduated centrifuge tube. 0.5 cc. of water is added followed by 0.5 cc. of a solution of sodium nitrite prepared by dissolving 15 gm. of potassium-free sodium nitrite (Merck) in 30 cc. of water. The contents of the tube are thoroughly mixed and allowed to stand for 5 minutes.<sup>2</sup> Water is added to 4 cc. and the contents are again mixed. 2 cc. of the sodium cobalti-nitrite reagent are added drop by drop. The contents of the tube are mixed and allowed to stand for a half hour, then centrifuged for 7 minutes at about 1,300 revolutions per minute. The precipitate will then be found at the bottom of the tube. All but 0.2 to 0.3 cc. of the supernatant fluid is removed. This is accomplished by means of the following apparatus. Through one opening of a two-holed cork is inserted a glass tube by means of which a positive pressure can be made in the centrifuge tube. Through the other hole is a tube which reaches to about 3 or 4 mm. above the precipitate. The lower end of this tube is drawn out to a

<sup>2</sup> If the sodium nitrite is not added, it will be found that the precipitate obtained on the addition of the cobalti-nitrite reagent will float on the surface of the fluid and adhere to the sides of the tubes. The precipitate will also adhere to the sides unless the tubes have been previously cleaned with the use of a brush, washed out with a strong cleaning fluid (commercial  $\text{H}_2\text{SO}_4$  and dichromate) and then thoroughly rinsed with distilled water. Low results will be obtained unless these procedures are carried out.

bore of about 1 mm. and curved so that the opening is directed upward. By fitting the cork to the centrifuge tube and blowing through the first opening the supernatant fluid can be readily removed without disturbing the precipitate. 5 cc. of water are allowed to run down the side of the tube which is then gently agitated so that the added water is mixed thoroughly with the residual reagent. Care should be taken that the precipitate itself is disturbed as little as possible. This may be accomplished by holding the tube vertically and gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the supernatant fluid. The tube is then centrifuged for 5 minutes. The procedure is repeated three times so that the precipitate is washed four times in all. The supernatant fluid from the last washing should be perfectly clear. After the removal of the fluid from the final washing the precipitate is ready to be titrated.

*Titration.*—An excess of 0.02 N potassium permanganate is added (1.6 to 2 cc. are sufficient for normal blood), followed by 1 cc. of approximately 4 N sulfuric acid. The precipitate is then thoroughly mixed with the fluid by means of a glass rod. The sample is heated in the boiling water bath for 45 to 60 seconds at the end of which time the solution should be clear and still pink. If all the precipitate is not oxidized, the contents will be cloudy and the intensity of the color will be seen to diminish. Heating should then be continued until the solution is clear but still pink. When the heating is continued too long, the contents again become cloudy and have a brownish color. If this is allowed to happen, the sample must be discarded as high results will be obtained. An amount of 0.01 N sodium oxalate sufficient to decolorize the solution completely (generally 2 cc.) is promptly added. The excess of oxalate is then determined by titrating to a definite pink color with 0.02 N potassium permanganate delivered from a micro-burette graduated in 0.02 cc.

The details for the calculation of the amount of potassium present in the sample and also the methods for the preparation of the reagents are given in a former paper (9).

For the determination of potassium in solutions of blood ash we placed a quantity of fluid equal to 0.1 or 0.2 cc. of blood in a graduated centrifuge tube, added water to 2 cc., and then

slowly added 1 cc. of the cobalti-nitrite reagent. The subsequent steps were the same as described for the determination of this element in serum (5).

*Calcium Method.*

4 cc. of the material prepared as outlined above are placed in a graduated centrifuge tube previously cleaned with commercial  $\text{H}_2\text{SO}_4$  and potassium dichromate. 1 cc. of saturated ammonium oxalate is added, followed by 2 cc. of a filtered saturated solution of sodium acetate. The contents are mixed and allowed to stand for 1 hour. The volume is made up to 8 cc. with distilled water, mixed, and then centrifuged for 15 minutes at about 1,300 revolutions per minute. This throws all the calcium oxalate precipitate to the bottom of the tube. All but 0.3 cc. of the supernatant fluid is removed by means of the apparatus described under the potassium method. The remaining fluid and the precipitate are mixed by tapping the tube. Enough 2 per cent ammonia (2 cc. of concentrated ammonia diluted to 100 cc.) is then added to bring the volume to 4 cc., care being taken to wash the sides of the centrifuge tube free from adherent oxalic acid. The tube is then centrifuged for 5 minutes. This procedure is repeated twice, thus making three washings in all. After the third washing the supernatant fluid is removed, the tube is shaken to suspend the precipitate, 2 cc. of approximately  $\text{N}$  sulfuric acid are added and the tube is warmed in the boiling water bath for a few minutes and titrated with 0.01  $\text{N}$  potassium permanganate until a definite pink color persists for at least 1 minute when viewed under a good light against a white background. The strength of the permanganate solution is determined by titrating against a 0.01  $\text{N}$  sodium oxalate (Sørensen).

The details for the calculation of the amount of calcium present in the sample and also the methods for the preparation of the reagents are given in a former paper (9).

For the determination of calcium on 0.1  $\text{N}$   $\text{HCl}$  solutions of blood ash the procedure is identical with that described above. The blood ash solution was made up so that 1 cc. corresponded to 1 cc. of blood. It was allowed to stand for 2 or 3 weeks to allow the sediment to settle. 2 cc. of this solution were used for each determination.

*The Magnesium Method.*

5 cc. of the supernatant fluid from the calcium determination are measured into a 30 cc. beaker, 1 cc. of  $(\text{NH}_4)_2\text{HPO}_4$  solution is added and then 2 cc. of concentrated ammonia. The next day the sample is filtered through a well packed Gooch crucible, washed ten times with 5 cc. of 10 parts of concentrated ammonia to 90 parts of water, then twice with 95 per cent alcohol made alkaline with ammonia. The crucible is returned to the beaker and dried for a few minutes at  $80^\circ \text{C}$ . in the oven.

10 cc. of 0.01 N HCl are added to the crucible and after a few hours the entire material is transferred to a test-tube, centrifuged, and 5 cc. of the supernatant fluid are measured into a flat bottomed colorimeter tube graduated for 10 cc., which contains 2 cc. of the iron thiocyanate solution. The volume is then made up to 10 cc. with 0.01 N HCl, a rubber stopper inserted, and the fluid mixed. A series of standards is prepared by adding varying amounts of a known  $\text{NH}_4\text{MgPO}_4$  solution in 0.01 N HCl, to 2 cc. samples of thiocyanate solution and bringing the volume up to 10 cc. as in the unknown samples. The color is compared by looking through the entire length of the liquid column against a white background.

*Calculation.*—The calculation is the same as in the original method: 
$$\frac{\text{Reading (cc. of standard solution)} \times 0.01 \times 2 \times 8/5 \times 100}{\text{cc. blood used in Ca determination}} = \text{mg. of magnesium in 100 cc. of blood.}$$

*Preparations of Reagents.*

1. *Ammonium Magnesium Phosphate Standard.*—This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ( $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$ ) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions (10).

2. *Ammonium Phosphate Solution*.—Ammonium phosphate solution is made as follows: 25 gm. of  $(\text{NH}_4)_2\text{HPO}_4$  are dissolved in 250 cc. of  $\text{H}_2\text{O}$ . 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc. This solution is diluted five times with water.

3. *The Ferric Thiocyanate Solution*.—This solution is made from two solutions which are mixed an hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 40 cc. with water.

4. *10 Per Cent Ammonia*.—100 cc. of concentrated ammonia are diluted to 1 liter.

### *Protocols.*

We have previously shown (5) that sodium may be precipitated quantitatively, as sodium pyroantimonate, from solutions of blood salts. Since the composition of the supernatant fluid after deproteinizing blood with trichloroacetic acid is comparable, except for the small amount of residual protein and the non-protein nitrogenous constituents, to some of the solutions of blood salts which we have analyzed, we have considered it unnecessary to repeat this demonstration here.

Table I shows that the sodium determinations when performed on the deproteinized solutions yield results practically identical with those obtained on a solution of the whole blood ash. The absolute values vary from 170 to 225 mg. of sodium per 100 cc. of blood.

Table II. The concentration of potassium seems to vary considerably in the blood of different animals. Bunge found 213 mg. of potassium in 100 cc. of pig's blood, 227 mg. in that of the horse, but only 34 mg. in the same volume of cow's blood and 20 mg. per 100 cc. of dog's blood. The lowest figure which Abderhalden reports is 21 mg. of potassium per 100 cc. of dog's blood while the highest figure is 227 mg. per 100 cc. of horse's blood. We have found that the potassium content of human



blood varies from 153 to 202 mg. of potassium per 100 cc. It varies with the percentage of corpuscles. It might be mentioned that the cobalti-nitrite reagent gives no precipitate when added to ferric chloride or trichloroacetic acid. We have shown elsewhere that none of the constituents of serum except potassium

TABLE I.  
*Sodium Determinations on Blood.*

Sample.	Plasma.	Na per 100 cc. of blood ashed.	Na per 100 cc of blood deproteinized.
	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
1	65	225	216
2	55	170	175
3	65	207	207
4	58	187	186
5	56	185	193
6	60	198	200
Average.....	60	195	196

TABLE II.  
*Potassium Determinations on Blood.*

Determinations on ashed blood.			Determinations on blood treated with trichloroacetic acid.		
Sample.	Plasma.	K per 100 cc. of blood.	Sample.	Plasma.	K per 100 cc of blood.
		<i>mg.</i>			<i>mg.</i>
1	61	172	7	61	180
2	60	187	8	62	175
3	57	188	9	57	202
4	68	153	10	59	193
5	58	186	11	65	164
6	57	200	12	65	169
			13	56	201
Average...	60	181		61	183

yields demonstrable amounts of insoluble nitrites with this reagent (6).

Table III. The concentration of calcium in serum or plasma is singularly constant (7). On the other hand the concentration of this element in blood varies inversely as the corpuscular con-

tent. The results which we obtained varied from 5.3 to 6.8 mg. of calcium per 100 cc. of blood. The individuals from whom the samples were obtained were all normal adults. We have found that the addition of ferric chloride to a solution of blood salts

TABLE III.  
*Calcium Determinations on Blood.*

Determinations on ashed blood.			Determinations on blood treated with trichloroacetic acid.		
Sample.	Plasma.	Ca per 100 cc. of blood.	Sample.	Plasma.	Ca per 100 cc of blood.
	<i>per cent</i>	<i>mg.</i>		<i>per cent</i>	<i>mg.</i>
1	58	5.3	8		6.3
2	57	5.3	9		5.3
3	72	6.7	10		6.1
4	59	6.2	11		5.3
5	58	5.3	12		5.7
6	65	5.9	13		6.4
7	57	5.5			
Average...		5.7			5.8

TABLE IV.  
*Magnesium Determinations on Blood.*

Determinations on ashed blood.		Determinations on blood treated with trichloroacetic acid.	
Sample	Mg. per 100 cc. of blood.	Sample.	Mg. per 100 cc. of blood.
	<i>mg</i>		<i>mg.</i>
1	2.8	5	2.6
2	2.8	6	4.0
3	3.8	7	3.8
4	2.3	8	3.8
Average.....	2.9		3.5

does not interfere with the quantitative determination of calcium. The results obtained on the deproteinized material and on the solutions of blood ash are practically identical.

Table IV. The concentration of magnesium in the blood of various animals has been found by Bunge and Abderhalden

to be fairly constant, varying only from 2 to 4 mg. per 100 cc. of blood (2). We have found that the concentration of this element in the blood of the adult male varies from 2.3 to 4.0 mg. per 100 cc.

#### CONCLUSIONS.

1. A method has been described by means of which sodium, potassium, calcium, and magnesium may be quantitatively determined on only 7 cc. of blood.

2. The basis of this method is deproteinization by means of trichloroacetic acid. The quantitative determination of each of these elements is then made on aliquots of the supernatant fluid by modifications of procedures recently described for serum.

3. The results obtained by these methods on deproteinized blood agree well with those obtained on solutions of blood ash.

4. We have found the concentration of these elements in 100 cc. of human blood to be as follows: sodium, 170 to 225 mg.; potassium, 153 to 201 mg.; calcium, 5.3 to 6.8 mg.; and magnesium, 2.3 to 4 mg.

5. The concentration of these elements in normal blood varies more than in normal serum. This is due to the variations in the corpuscular content of the blood.

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# PHOSPHORIC ESTERS OF SOME SUBSTITUTED GLUCOSIDES AND THEIR RATE OF HYDROLYSIS.

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The purpose of the preparation of the substance to be described in this communication was developed in a previous publication by Levene and Yamagawa. The interest, as was explained, is both of a theoretical and a practical nature. The question to be answered is the following. Does the stability of the inorganic radicle in a phosphoric ester of a sugar (free or substituted) depend on the position of the inorganic radicle?

The answer to this question is given in the following table of the constants of hydrolysis of three 1-2-acetone phosphoric acid glucoses.

	K
1-2-Monoacetone phosphoric acid glucose.....	44 ( $10^{-3}$ )
1-2-Monoacetone-6-phosphoric acid glucose.....	58 ( $10^{-3}$ )
1-2-Monoacetone-3- or 5-phosphoric acid glucose <sup>1</sup> .....	24 ( $10^{-3}$ )

Substance 1 was prepared from 1-2-acetone glucose, hence the position of the phosphoric acid in it may be in any position from 3 to 6 (inclusive). Substance 2 was prepared in the course of preparation of the derivative from 1-2-3-5-diacetone, hence it is in the main 1-2-diacetone-6-phosphoric acid glucose. The third substance was prepared from 1-2-3-5-diacetone-6-benzoyl glucose. This was converted into 1-2-acetone-6-benzoyl glucose, this further into 1-2-acetone-3- or 5-phosphoric acid-6-benzoyl glucose, and finally the latter into 1-2-acetone-3- or 5-phosphoric

<sup>1</sup> The second sentence of the last paragraph on p. 324, *J. Biol. Chem.*, xliii, should read: Whereas the fourth was obtained by the action of phosphorus oxychloride on monoacetone glucose, the fifth is formed as a by-product by the action of phosphorus oxychloride on diacetone glucose.

acid glucose. Thus in Substance 2 the phosphoric acid radicle is attached to carbon atom 6, and in Substance 3 in position 3 or 5. The differences in the rate of hydrolysis are far beyond the limits of error of the method. It is interesting to note that the rate of hydrolysis of Substance 3 is practically the same as found in the earlier experiments of Levene and Yamagawa on 1-2-acetone-6-benzoyl phosphoric acid. This similarity is easily understood, since the benzoyl group being in position 6 is very labile, hence, in the course of hydrolysis the benzoyl derivative is soon transformed into the monoacetone phosphoric acid glucose. In the same manner is explained the fact that 1-2-acetone-6-phosphoric acid glucose, and 1-2-3-5-diacetone-6-phosphoric acid hydrolyze with the same velocity.

The analytical data on the barium salts of phosphoric esters, here reported, call for a special note. They are not so satisfactory as is desired. However, it has been the experience of this laboratory that when barium salts of pure phosphoric esters are obtainable only in an amorphous form, the analytical results are often not perfect. Thus it was found practically impossible to obtain a perfect agreement between the found and required elementary composition of the amorphous barium salt prepared from the crystalline adenosinphosphoric acid.

The observations on the phosphoric esters of the 1-2-3-5-methyl glucose and of the 1-3-5-6-methyl glucose are in harmony with those on the esters of the 1-2-acetone glucose.

Thus the rate of hydrolysis of 1-2-3-5-methyl-6-phosphoric acid glucose proceeded normally, giving an average ratio,  $K = 44 (10^{-3})$ , which is not far removed from that of the acetone glucose ester having the inorganic radicle in position 6, where  $K = 56 (10^{-3})$ . On the other hand, the rate of hydrolysis of 1-3-5-6-methyl-2-phosphoric acid glucose proceeded abnormally. There was noted a rapid hydrolysis at the beginning which reached a maximum after about 6 hours, and the further progress was very slow. The explanation of this result may be the following. The 1-3-5-6-methyl glucose is apparently very difficult to prepare in pure form. The material employed for coupling with phosphoric acid may have contained as an impurity some trimethyl glucose with either carbon atom 1 or carbon atom 6 free. Thus the material derived from this product may be a mixture of two

phosphoric esters, one with an inorganic radicle in position 1 or 6, which is labile, and the other in position 2 which is very stable. The first is decomposed rapidly, whereas the second proceeds at the low rate indicated in the table.

The estimations of the rate of hydrolysis were carried out by Miss I. Weber.

#### EXPERIMENTAL.

##### *$\alpha$ -Methyl Glucosidophosphoric Acid.*

Portions of 10 gm. of dried methyl glucoside are dissolved in 50 cc. of warm, water-free pyridine. This solution is cooled to  $-20^{\circ}\text{C}$ . and to it is added, in small portions, a solution of 7.8 gni. (1 mol) phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-20^{\circ}\text{C}$ .

It is important that the pyridine as well as the methyl glucoside should be absolutely dry. For this purpose the substance is dried over phosphorus pentoxide at  $100^{\circ}\text{C}$ . under a pressure of about 1 mm. The pyridine is boiled with reflux over barium oxide for 5 hours and then distilled. The pyridine and phosphorus oxychloride should be well cooled before mixing, and if the pyridine is dry no pyridine hydrochloride will settle out even after standing for some time.

After the phosphorus oxychloride in pyridine has been added to the solution of the glucoside, the reaction mixture is allowed to remain at  $-20^{\circ}\text{C}$ . for several hours, during which time a considerable amount of pyridine hydrochloride separates. The reaction mixture is now diluted with 20 cc. of ice water and allowed to come to room temperature when it is further diluted by pouring it into 200 cc. of cold water. An excess of barium hydrate (100 gm.) is added and the pyridine removed by distillation under diminished pressure. The temperature of the bath should not be above  $+30^{\circ}\text{C}$ .

When all the pyridine has been removed the solution is made just acid to Congo red with sulfuric acid. The hydrochloric acid is now removed by the addition of 30 gm. of silver sulfate. After shaking this mixture for half an hour it is filtered and the silver removed from the filtrate by hydrogen sulfide and the latter removed by a current of air.

An excess of barium hydrate is added and carbon dioxide passed into the solution until the reaction is neutral to litmus. The solution is now filtered and concentrated under diminished pressure and low temperature. When the volume has been reduced to one half of the original, the solution is filtered and the filtrate concentrated to a small volume which is then poured into a large volume of absolute alcohol. For purification the precipitate is dissolved in a small quantity of water, filtered, and reprecipitated with alcohol. The yield was 9.0 gm.

For analysis the substance is dried under diminished pressure at the temperature of water vapor.

0.1042 gm. of substance gave on combustion 0.0866 gm. of  $\text{CO}_2$  and 0.0310 gm. of  $\text{H}_2\text{O}$ .

0.2757 gm. of substance gave 0.0746 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0982 " " " 0.0485 " "  $\text{BaSO}_4$ .

$\text{C}_7\text{H}_{13}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 30.7, H 4.8, P 11.0.

Found (calculated Ba-free). C 31.8, H 4.68, P 10.6.

The optical rotation in water was found:

$$[\alpha]_{\text{D}}^{20} = \frac{+1.32^\circ \times 100}{2 \times 1.615} = +81.8^\circ$$

### *1-2-3-5-Diacetone Glucose.*

Fischer's latest method for the preparation of diacetone from  $\beta$ -glucose<sup>2</sup> in a somewhat modified form was employed.

75 gm. of dried  $\beta$ -glucose, which is readily prepared by the method of Behrend, are placed in an ordinary glass-stoppered acid bottle with 1,500 cc. of dried acetone containing 1 per cent hydrochloric acid. The bottle was shaken for 24 hours at  $30^\circ\text{C}$ . The contents are now dark-colored and nearly all the  $\beta$ -glucose has dissolved. The solution is filtered and neutralized to litmus with sodium methyrate, which is prepared by dissolving 4 gm. of sodium in 400 cc. of dry methyl alcohol. When neutral the acetone solutions assume a much lighter color.

The solution is again filtered and concentrated to dryness under diminished pressure. A solid cake is formed and repeatedly extracted with warm petroleum ether from which on cooling, diac-

<sup>2</sup> Fischer, E., and Rund, C., *Ber. chem. Ges.*, 1916, xlix, 93.

tone glucose crystallizes. The diacetone glucose is further purified by the method described by Fischer. 600 gm. of  $\beta$ -glucose under proper conditions yield 350 gm. of diacetone glucose.

Although some monoacetone is also obtained the yield of this substance is comparatively small.

*1-2-3-5-Diacetone-6-Phosphoric Acid Glucoside.*

The action of phosphorus oxychloride on diacetone glucose depending upon certain conditions yields either a diacetone glucose phosphoric acid, the barium salt of which is soluble in absolute alcohol, or a monoacetone phosphoric acid, the barium salt of which is insoluble in alcohol.

10 gm. of thoroughly dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-35^{\circ}$ . This low temperature is readily obtained by using a mixture of  $\text{CaCl}_2 + 6\text{H}_2\text{O}$  and ice. To the sugar solution are added in small amounts 5.4 gm. phosphorus oxychloride in 20 cc. of dry pyridine, likewise cooled to  $-35^{\circ}$ . The mixture is now transferred to a bath of  $-10^{\circ}$  and kept therein for 2 hours. During this time crystals of pyridine hydrochloride have separated. The reaction mixture is again cooled to  $-35^{\circ}\text{C}$ . and about 20 cc. of moist pyridine, also cooled to  $-35^{\circ}\text{C}$ ., added in such small quantities that the temperature will not rise above  $-10^{\circ}$ . This is then followed by the addition of a cold solution of 10 cc. each of pyridine and water and finally a small cake of ice.

The reaction product is now allowed to come to room temperature. An excess of barium hydrate is added, the pyridine removed under diminished pressure, and the hydrochloric acid removed by shaking with silver sulfate, and further treated as previously described.

The barium salt of diacetone phosphoric acid is very soluble in alcohol and is not precipitated by ether or acetone. On allowing the alcoholic solution to evaporate to dryness the barium salt is obtained as a fine white powder which when dry is no longer hygroscopic. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried to constant weight under diminished pressure at the temperature of water vapor.



0.1133 gm. of substance gave on combustion 0.1331 gm. of  $\text{CO}_2$  and 0.0482 gm. of  $\text{H}_2\text{O}$ .

0.0923 gm. of substance gave 0.0341 gm. of  $\text{BaSO}_4$ .

0.2770 " " " " 0.0625 " "  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{12}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 42.25, H 6.18, P 9.10.

Found (calculated Ba-free). C 41.5, H 6.18, P 8.90.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-11^\circ \times 100}{1 \times 4.472} = -2.48^\circ$$

*1-2-Monoacetone Phosphoric Acid Glucoside from Diacetone Glucose.*

This substance is obtained by the action of phosphorus oxychloride on diacetone glucose when the temperature of the reaction mixture is allowed to rise above  $+10^\circ$  during the process of destroying the unutilized phosphorus oxychloride.

10 gm. of dried diacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-20^\circ$ . To this is added 5.4 gm. of phosphorus oxychloride dissolved in 20 cc. of pyridine also cooled to  $-20^\circ$ . The mixture is allowed to remain at  $-20^\circ$  for several hours and then 20 cc. of ice cold water are added. The temperature of the mixture rises to about  $+30^\circ$ . After allowing it to stand at room temperature for a while it is further diluted with water. An excess of barium hydrate is added and the pyridine removed by distillation under diminished pressure.

The product is treated with silver sulfate and barium hydrate as previously described. The final residue is soluble in water which on the addition of alcohol forms a gelatinous mass. The barium salt is precipitated from its aqueous solution by pouring it into a large volume of dry acetone. The salt is obtained as a fine white powder. It does not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1032 gm. of substance gave on combustion 0.1016 gm. of  $\text{CO}_2$  and 0.0394 gm. of  $\text{H}_2\text{O}$ .

0.2679 gm. of substance gave 0.0653 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0893 " " " " 0.0424 " "  $\text{BaSO}_4$ .

$\text{C}_9\text{H}_{16}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.9, H 5.61, P 9.30.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.35^\circ \times 100}{1 \times 5.116} = +6.8^\circ$$

*1-2-Monoacetone Phosphoric Acid Glucoside from Monoacetone Glucose.*

10 gm. of dry monoacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this is added 4.4 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine. The reaction mixture is allowed to stand for some time at  $-20^\circ$  and treated as described in the previous preparations. The residue is soluble in 95 per cent alcohol which on pouring into a large excess of dry ether precipitates the barium salt of monoacetone phosphoric acid glucoside. The yield was 9 gm.

The substance is dried for analysis under diminished pressure at the temperature of xylene vapor.

0.1103 gm. of substance gave on combustion 0.1056 gm. of  $\text{CO}_2$  and 0.0400 gm. of  $\text{H}_2\text{O}$ .

0.2940 gm. of substance gave 0.0744 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0980 " " " " 0.0438 " "  $\text{BaSO}_4$ .

$\text{C}_9\text{H}_{16}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.4, H 5.42, P 9.55.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{+20^\circ \times 100}{1 \times 4.000} = +5.0^\circ$$

*1-2-Monoacetone- $\beta$ -Benzoyl Phosphoric Acid Glucoside.*

The benzoyl monoacetone glucose was prepared from benzoyl diacetone glucose according to the method of Fischer.

10 gm. of dried benzoyl monoacetone glucose are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this are added in small amounts 4.7 gm. of phosphorus oxychloride dissolved in 20 cc. of dry pyridine, also cooled to  $-30^\circ$ . The mixture is allowed to stand at  $-30^\circ$  for several hours during which time crystals of pyridine hydrochloride have separated. Moist pyridine, cooled to  $-20^\circ$ , is now added, followed by a cake of ice. The mixture is allowed to come to room temperature, diluted with water,

and treated as previously described. The residue is soluble in absolute alcohol from which the barium salt is precipitated by pouring it into a large volume of dry ether. The yield was 10 gm.

For analysis the substance was dried under diminished pressure at the temperature of chloroform vapor.

0.1097 gm. of substance gave on combustion 0.1316 gm. of  $\text{CO}_2$  and 0.0426 gm. of  $\text{H}_2\text{O}$ .

0.2941 gm. of substance gave 0.0642 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0980 " " " " 0.0394 " "  $\text{BaSO}_4$ .

$\text{C}_{16}\text{H}_{20}\text{O}_7\text{H}_2\text{PO}_3$ . Calculated. C 47.4, H 4.95, P 7.64.

Found (calculated Ba-free). C 42.9, H 5.3, P 8.06.

The optical rotation was found:

$$[\alpha]_D^{20} = \frac{+0.26^\circ \times 100}{1 \times 2.184} = +11.94^\circ$$

From the analysis of the substance and from the fact that it is non-reducing it was supposed that in the process of preparation a small part of the benzoic acid radicle was removed. This assumption was corroborated by the estimation of the benzoic acid on hydrolysis of the substance.

1.000 gm. of barium salt was hydrolyzed with dilute sulfuric acid. The solution was extracted with ether, the ethereal solution dried with anhydrous sodium sulfate and concentrated. The residue consisted of benzoic acid and weighed 0.1376 gm. or 13.76 per cent. Theory required 22.6 per cent.

*1-2-Monoacetone Phosphoric Acid Glucose from*

*1-2-Monoacetone-6-Benzoyl Phosphoric Acid Glucose.*

15 gm. of the barium salt of the phosphorated sugar were dissolved in 150 cc. of water and made just acid to Congo red with sulfuric acid. An additional 10 cc. of 2 N sulfuric acid were added and all made up to 200 cc. This was heated in a water bath at  $50^\circ\text{C}$ . for 70 minutes. The solution was cooled, filtered, and extracted with ether. The aqueous solution was made alkaline with barium hydrate and filtered from the barium phosphate. Carbon dioxide was passed until the reaction was neutral to litmus. The solution was again filtered and concentrated under diminished pressure at  $30^\circ\text{C}$ . The residue was soluble in 95 per

cent alcohol and the barium salt precipitated by pouring it into a large volume of dry ether. The yield was 6 gm.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1091 gm. of substance gave on combustion 0.0979 gm. of  $\text{CO}_2$  and 0.0420 gm. of  $\text{H}_2\text{O}$ .

0.2811 gm. of substance gave 0.0658 gm. of  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.0937 " " " 0.0446 " "  $\text{BaSO}_4$

$\text{C}_9\text{H}_{16}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 35.75, H 6.00, P 10.28.

Found (calculated Ba-free). C 35.67, H 6.24, P 9.48.

The substance does not reduce Fehling's solution until after hydrolysis with dilute acid.

The optical rotation found was:

$$[\alpha]_D^{20} = \frac{0.25^\circ \times 100}{1 \times 0.4000} = +6.25^\circ$$

### *2-3-5-Trimethyl-Methyl Glucoside.*

The trimethyl glucoside was prepared by the method of Haworth.<sup>3</sup> Methyl glucoside was methylated in portions of 20 gm. each. After 300 gm. of glucoside had been methylated the combined product consisting of a mixture of the methylated glucosides was subjected to a fractional distillation. The progress of fractionation was controlled by means of the index of refraction as well as by methoxy determinations.

The product was separated into the following fractions:

Fraction.	$n$	$N_D^{20}$	$\text{CH}_3\text{O}$	Boiling point $P = 0.15 \text{ mm.}$
			<i>per cent</i>	$^\circ\text{C.}$
I	46°56.5'	1.4449	60.95	85
II	46°51.5'	1.4450	61.08	90
III	46°41.5'	1.4446	60.00	95
IV	46°01.5'	1.4502	56.67	105
V	45°11.5'	1.4550	52.30	110

The theory for tetramethyl-methyl glucoside requires 61.75 per cent  $\text{CH}_3\text{O}$  and trimethyl-methyl glucoside calls for 52.54

<sup>3</sup> Haworth, W. N., *J. Chem. Soc.*, 1915, cvii, 8.

per cent  $\text{CH}_3\text{O}$ . Fractions IV and V were again distilled, the main fraction boiling at  $108^\circ\text{C}$ .,  $P = 0.07$  mm.,  $n = 44^\circ 43'$ ,  $N_D^{20} = 1.45786$ ,  $D_4^{20} = 1.1477$ . Found  $M_p = 56.06$ .

Theory requires 55.917. The yield of this fraction was 137 gm. Methoxy determination:

0.1803 gm. of substance gave 0.7145 AgI (factor = 13.2) = 52.30 per cent of  $\text{CH}_3\text{O}$ . Theory requires 52.54 per cent.

*2-3-5-Trimethyl-6-Phosphoric Acid Methyl Glucoside.*

Portions of 10 gm. of the glucoside are dissolved in 50 cc. of dry pyridine and cooled to  $-30^\circ$ . To this mixture is added a solution of 6.5 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-30^\circ\text{C}$ .

The reaction is rather feeble and only after some time pyridine hydrochloride begins to separate. The reaction mixture is allowed to stand for several hours at  $-20^\circ$  and then 20 cc. of cold moist pyridine are added. The product is now allowed to come to room temperature and further diluted with ice cold water.

After making alkaline with 60 gm. of solid barium hydrate, the pyridine is removed by distillation under diminished pressure. The reaction product is then treated with silver sulfate and barium hydrate.

The barium salt of this substance is very soluble in alcohol and acetone. It was purified by dissolving it in a small quantity of acetone and precipitating it in a large volume of dry ether. The salt is obtained as a white hygroscopic powder. For analysis it is dried under diminished pressure at the temperature of xylene vapor.

0.1122 gm. of substance gave on combustion 0.0462 gm. of  $\text{H}_2\text{O}$  and 0.1164 gm. of  $\text{CO}_2$ .

0.0908 gm. of substance gave 0.0382 gm. of  $\text{BaSO}_4$ .

0.2723 " " " " 0.0705 " "  $\text{Mg}_2\text{P}_2\text{O}_7$ .

$\text{C}_{10}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_5$ . Calculated. C 37.97, H 6.68, P 9.80.

Found (calculated Ba-free). C 37.50, H 6.12, P 9.45.

The rotation in water was found:

$$[\alpha]_D^{20} = \frac{-2.36^\circ \times 100}{1 \times 3.062} = +77.07^\circ$$

*3-5-6-Trimethyl-1-2-Acetone Glucose.*

3-5-6-Trimethyl-methyl glucoside was prepared by methylating 1-2-acetone glucose and converting the product into the glucoside. The methylation of monoacetone glucose had been attempted by Irvine and Scott<sup>4</sup> using the silver oxide and methyl iodide method. The results by this method are not satisfactory. Attempts to methylate the trimethyl glucose obtained after cleaving off the acetone were also unsuccessful. The following method was finally adopted.

Portions of 25 gm. of monoacetone glucose were methylated with an excess of dimethyl sulfate by the method of Haworth. The temperature of the bath should not exceed 55°; the methylation should proceed rather slowly, and the alkali should be always present in a slight excess. To 25 gm. of monoacetone there were used 150 cc. of 30 per cent sodium hydroxide and 90 cc. of freshly distilled dimethyl sulfate. After all the reagents had been added the stirring was continued for one hour at the same temperature. The solution was then cooled and ammonium hydroxide added to destroy any unutilized dimethyl sulfate. The solution was extracted with chloroform and the chloroform extract, after drying over anhydrous sodium sulfate, concentrated under diminished pressure to a syrup. The product of five such methylations was combined, dissolved in ether, dried with anhydrous sodium sulfate, and subjected to a fractional distillation.

The largest fraction amounting to 90 gm. boiled at 88–90° at a pressure of 0.03 mm.

0.1025 gm. of substance gave on combustion 0.2042 gm. of CO<sub>2</sub> and 0.0780 gm. of H<sub>2</sub>O.

C <sub>12</sub> H <sub>22</sub> O <sub>6</sub> .	Calculated.	C 54.96, H 8.17.
	Found.	C 54.33, H 8.5.

The optical rotation was:

$$[\alpha]_D^{20} = \frac{-1.15^\circ \times 100}{1 \times 4.034} = -28.5^\circ$$

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<sup>4</sup> Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 573.

*3-5-6-Trimethyl Glucose.*

Hydrolysis of the trimethyl acetone glucose was carried out in 75 per cent alcoholic solution containing 0.5 per cent hydrochloric acid and heating for 1 hour at 100°. The solution was shaken with silver carbonate, extracted with ether, and the ethereal solution dried and concentrated to a syrup. This was distilled and a fraction obtained boiling at 147° and  $P = 0.05$  mm.

0.1150 gm. of substance gave on combustion 0.2080 gm. of  $\text{CO}_2$  and 0.0872 gm. of  $\text{H}_2\text{O}$ .

$\text{C}_9\text{H}_{19}\text{O}_6$ . Calculated. C 48.75, H 8.11.

Found. C 49.32, H 8.43.

The optical rotation in alcohol was:

$$[\alpha]_D^{20} = \frac{\text{Initial. } -0.75^\circ \times 100}{1 \times 6.874} = -10.95^\circ \quad [\alpha]_D^{20} = \frac{\text{Final. } -1^\circ \times 100}{1 \times 6.874} = -14.6^\circ$$

It was attempted to convert this product into its methyl glucoside by all the known methods and by several modifications as regards the concentration of catalyst and time of reaction. In every experiment the unchanged material was recovered.

*3-5-6-Trimethyl-Methyl Glucoside.*

The conversion of trimethyl monoacetone glucose to the glucoside was obtained finally by the following process:

50 gm. of trimethyl acetone glucose were dissolved in 100 cc. of dry methyl alcohol containing 0.1 per cent hydrochloric acid and heated in sealed tubes for 24 hours at 100°C. The hydrochloric acid was removed by shaking with moist silver carbonate and the solution concentrated under diminished pressure to a syrup. This was taken up in ether and dried with anhydrous sodium sulfate. The ethereal solution was concentrated and the syrup fractionated. The larger part distils at 135°,  $P = 0.035$  mm. The yield was 20 gm.

0.1510 gm. of substance gave on combustion 0.2758 gm. of  $\text{CO}_2$  and 0.1174 gm. of  $\text{H}_2\text{O}$ .

$\text{C}_{10}\text{H}_{21}\text{O}_6$ . Calculated. C 50.85, H 8.47.

Found. C 49.8, H 8.7.

Repeated attempts to obtain this glucoside uncontaminated with traces of free sugar were unsuccessful. The product always produced a slight reduction of Fehling's solution.

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

10 gm. of 3-5-6-trimethyl-methyl glucoside were dissolved in dry pyridine, cooled to  $-20^{\circ}$ , and a solution of 5.1 gm. of phosphorus oxychloride in 20 cc. of pyridine, also cooled to  $-20^{\circ}$ , was slowly added. There was a slight rise in temperature and after standing at  $-10^{\circ}$  for some time, crystals of pyridine hydrochloride separated. The barium salt, prepared by the method previously outlined, was found to be soluble in alcohol, ether, and acetone. The alcoholic solution was allowed to evaporate. The barium salt which was obtained as a white powder did not reduce Fehling's solution until after hydrolysis with acid.

For analysis the substance was dried under diminished pressure at the temperature of water vapor.

0.1070 gm. of substance gave on combustion 0.1258 gm. of  $\text{CO}_2$  and 0.0570 gm. of  $\text{H}_2\text{O}$ .

0.2918 gm. of substance gave 0.0717 gm. of  $\text{Mg}_3\text{P}_2\text{O}_7$ .

0.0973 " " " " 0.0274 " "  $\text{BaSO}_4$ .

$\text{C}_{10}\text{H}_{19}\text{O}_6\text{H}_2\text{PO}_3$ . Calculated. C 37.97, H 6.68, P 9.80.

Found (calculated Ba-free). C 38.6, H 7.15, P 8.25.

The barium salt was in the main an acid barium salt, since analysis showed 16.5 per cent of barium; theory requires for the acid salt 17.85 per cent, and for the neutral salt 30.5 per cent barium.

The optical rotation in water was:

$$[\alpha]_D^{20} = \frac{0.71^{\circ} \times 100}{1 \times 2.692} = +26.38^{\circ}$$

*Rates of Hydrolysis.*

*1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.*

1.658 gm. of the barium salt of this substance were dissolved in a small volume of warm water and made up to 50 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 2.832 cc. of 0.1 N  $\text{H}_2\text{SO}_4$  and 0.168 cc. of water and sealed. The tubes were heated in an oil bath at 100 cc.



for the intervals indicated in the following tables. The method of analysis was as described in the paper of Levene and Yamagawa.<sup>5</sup>

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

3.651 gm. of the barium salt of this substance were dissolved in a little warm water and made up to 25 cc. Of this solution 3 cc., equivalent to 0.030 gm. of P, were put into glass tubes together with 1.227 cc. of 0.1 N H<sub>2</sub>SO<sub>4</sub> and 0.773 cc. of water and sealed. The tubes were heated at 100° for the intervals indicated in the following tables.

*1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.*

Time.	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	Average.	P in Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .	P in free acid.	P of total P.
hrs.	gm.	gm.	gm.	per cent	per cent
1	0.0014	0.0013	0.0009	0.27	3.02
	0.0012				
2	0.0030	0.0032	0.0022	0.67	7.42
	0.0034				
4	0.0062	0.0063	0.0044	1.33	14.61
	0.0061				
6	0.0082	0.0083	0.0059	1.75	19.25
	0.0083				
8	0.0099	0.0100	0.0070	2.11	23.19
	0.0100				
16	0.0160	0.0161	0.0115	3.47	38.21
	0.0162				
24	0.0199	0.0199	0.0139	4.20	46.16
	0.0198				

<sup>5</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, 1920, xliii, 323.

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

Time.	Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	Average.	P in Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	P in free acid.	P of total P.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
1	0.0178	0.0179	0.0125	3.37	41.53
	0.0179				
2	0.0210	0.0213	0.0151	4.09	50.31
	0.0214				
4	0.0210	0.0210	0.0146	3.96	48.71
	0.0209				
6	0.0232	0.0233	0.0162	4.39	54.05
	0.0234				
8	0.0207	0.0208	0.0145	3.92	48.25
	0.0208				
16	0.0212	0.0213	0.0148	4.01	49.41
	0.0214				

## Sample 2.

1	0.0303	0.0302	0.0084	4.46	70.04
	0.0301				
2	0.0303	0.0305	0.0085	4.50	70.74
	0.0307				
4	0.0320	0.0324	0.0090	4.89	76.90
	0.0328				
6	0.0348	0.0346	0.0096	5.11	80.26
	0.0344				

*1-2-Monoacetone-3- or 5-Phosphoric Acid Glucose.*

<i>T</i>	$\text{Mg}_2\text{P}_2\text{O}_7$ ( <i>x</i> )	<i>a-x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0033	0.1044	0.00023
120	0.0080	0.0997	0.00028
240	0.0157	0.0920	0.00028
360	0.0208	0.0869	0.00026
480	0.0250	0.0827	0.00024
960	0.0402	0.0675	0.00021
1,440	0.0500	0.0577	0.00020
Average .....			0.00024

$$a = 0.1077$$

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

<i>T</i>	$\text{Mg}_2\text{P}_2\text{O}_7$ ( <i>x</i> )	<i>a-x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0448	0.0629	0.0038
120	0.0530	0.0547	0.0024
240	0.0525	0.0552	0.0012
360	0.0583	0.0494	0.0009
480	0.0520	0.0557	0.0006
960	0.0533	0.0533	0.0003
1,440	0.0692	0.0385	0.0003

$$a = 0.1077$$

*3-5-6-Trimethyl-2-Phosphoric Acid Methyl Glucoside.*

## Sample 2.

<i>T</i>	$\text{Mg}_2\text{P}_2\text{O}_7$ ( <i>x</i> )	<i>a-x</i>	$\frac{1}{t} \log \frac{a}{a-x}$
<i>min.</i>	<i>gm.</i>		
60	0.0755	0.0322	0.0087
120	0.0763	0.0314	0.0044
240	0.0829	0.0248	0.0026
360	0.0865	0.0212	0.0017

$$a = 0.1077$$

## THE QUANTITATIVE DETERMINATION OF AMINO-ACIDS OF FEEDS.

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### INTRODUCTION.

The need and the importance of knowledge concerning the amino-acid content of foods and feeds require no emphasis. Grindley, Joseph, and Slater (1) in 1915 were the first to publish data on the quantitative determination of amino-acids in feeds. About 1 month later Nollau (2) published results on the amino-acid contents of certain commercial feeds. Later in the same year Grindley and Slater (3), in a second paper, published additional results on the same problem. Both Grindley and associates and Nollau used in their work the Van Slyke method, but the results from the two laboratories do not agree well. As is stated in more detail later, the lack of concordant results, in general, may be explained by differences in the procedures used.

Criticism has been made, however, of the application of a method, designed entirely for the purpose of analyzing pure isolated proteins, to the analysis of heterogeneous mixtures such as feeds. Among several difficulties mentioned, the effects of the non-protein nitrogenous constituents and of the carbohydrates on the results of the Van Slyke analysis were unknown. In order to determine the effect of the non-protein nitrogenous material, Grindley and Eckstein (4) made a study of the non-protein constituents extracted from various feeds with cold water. Hart and Bentley (5) made a similar study but used hot water instead of cold water as their extracting fluid. From the fact that most of the non-protein nitrogen of the feeds examined was in the form of ammonia or  $\alpha$ -amino-acid nitrogen, free or combined, Grindley and Eckstein in part conclude:

“ . . . it seems quite evident that only a small part, if any, of the nonprotein nitrogenous constituents of foods and feeding stuffs can in anyway interfere with the application of the Van Slyke method for the determination of the chemical groups characteristic of the different amino acids of protein to the estimation of the free and combined amino acids and amides of feeding stuffs.”

And that no claim to perfection is made for the results published by Grindley and his associates is shown by the statement of Grindley (6): “Further it is also quite evident that the results so far obtained in this work are only approximately accurate and at present are to be considered of comparative value only.”

The chief source of error in the method of analysis used by Grindley and associates was thought to be caused by the presence of the carbohydrates in the feeds during the hydrolysis and subsequent analysis. Gortner and his associates (7, 8) have made an extensive study of the formation of humin in the presence of carbohydrates during acid hydrolysis. These authors have shown that the formation of humin depends to a large extent on the presence of carbohydrates and that the quantity of humin formed on hydrolysis of pure proteins is greatly increased by the addition of carbohydrate material. Attempting to reduce the quantity of humin formed during hydrolysis, Eckstein and Grindley (9) made two decided improvements on the older method. The first was the removal of some of the non-protein nitrogenous constituents by extractions with ether and cold absolute alcohol. The second was “the conversion, as far as possible, of the insoluble carbohydrates into soluble carbohydrates by boiling the feeding-stuffs with 0.1 per cent hydrochloric acid.” In this manner it was possible to separate a large part of the carbohydrates from the main portion of the proteins before the latter are hydrolyzed. The quantity of humin nitrogen obtained by this method compared very favorably with that formed in the analysis of some of the pure proteins.

While this method of Eckstein and Grindley was a decided improvement over the first method used by Grindley and associates, it was far from perfect. The perfection of a method for the quantitative determination of the amino-acid content of feeds has been the aim of an extensive investigation conducted in this laboratory.

*Method.*

The method outlined below is the culmination of 146 experiments designed to separate the proteins of feeds quantitatively, either as such or as hydrolyzed proteins, from the other constituents of the sample which would interfere with the determination of amino-acids by the Van Slyke method (10,11). The completeness with which this has been accomplished is shown by an examination of Tables I and II.

The analysis is divided into two distinct parts: First, the treatment of a sample of feed so that all the proteins are obtained in solutions sufficiently free from interfering substances so that the Van Slyke method for the estimation of certain amino-acids may be applied; and second, the quantitative estimation of these amino-acids.

The first part of the procedure consists of a series of extractions with various solvents, and, in those extracts in which it is necessary, the separation of the proteins, by various methods, from the interfering substances. The residue remaining after the last extraction consists chiefly of crude fiber and contains very little nitrogen. The essential features of this part of the procedure are as follows:

1. The non-protein nitrogenous constituents are extracted from a weighed quantity of the finely ground feed, equivalent to approximately 6 gm. of protein, by extracting with anhydrous ether, cold absolute ethyl alcohol, and cold 1.0 per cent trichloroacetic acid, in the order named. These extractions as well as all other extractions in the cold are carried out in the following manner: The sample is placed in a 500 cc. centrifuge bottle, 100 to 200 cc. of the extracting liquid are added, and the bottle is placed on a shaker arrangement which rolls the bottle back and forth continuously. Usually but two extractions are made each 24 hours; one extraction for a 7 to 8 hour period is made during the day and a second extraction for a 14 to 15 hour period is made during the night. As a rule six or seven extractions with each solvent are necessary to insure complete extractions in the cold. After each extraction period the solution is centrifuged and the supernatant liquid decanted.

The small amount of protein extracted by the cold trichloroacetic acid is recovered by precipitation with colloidal ferric hydrate.

2. The main portion of the proteins is next extracted with cold dilute sodium hydroxide solution on the shaker. A 0.2 per cent solution of sodium hydroxide is used as the extracting liquid during the day and a 0.1 per cent solution is used during the longer night period. After the sixth extraction with alkali the residue is washed a few times with 100 cc. portions of ammonia-free water to remove the alkali.

3. The starch is next removed from the residue by extracting with hot 2.0 per cent trichloroacetic acid. The residue from the dilute alkali extraction is transferred to a round bottom digestion flask with 500 cc. of 2.0 per cent trichloroacetic acid. The flask is placed on a boiling steam bath and with frequent shakings allowed to digest until it is apparent (from the disappearance of the milky color) that much of the starch has been dissolved. The solution is then filtered, washed with hot water, and the residue treated again with 250 cc. of the 2.0 per cent trichloroacetic acid. A third digestion with 250 cc. of the acid generally completes the extraction of starch. A small amount of protein is extracted by the trichloroacetic acid and this is separated from the starch by concentrating the united trichloroacetic acid filtrates under diminished pressure to about one-third their original volume and precipitating the starch with two volumes of alcohol. Only a negligible amount of nitrogen is found in the starch thus precipitated, while the filtrate is practically starch-free.

4. The residue from the above treatment is next boiled with 250 cc. of 20 per cent hydrochloric acid for 3 minutes, cooled, filtered, and washed with ammonia-free water (keeping the washings separate from the 20 per cent HCl filtrate). The residue is again treated with 250 cc. of 20 per cent HCl in exactly the same manner as before.

5. The residue from the 20 per cent HCl treatment is transferred to a centrifuge bottle and extracted for three 24 hour periods on the shaker with 50 cc. portions of 5 per cent sodium hydroxide solution.

The entire protein content of the sample is found, from the above procedure, in the following fractions: (a) the colloidal ferric hydrate precipitate from the cold 1.0 per cent trichloroacetic acid extract; (b) the dilute alkali extract; (c) the filtrate from the alcoholic precipitation of the starch; (d) the 20 per cent hydro-

chloric acid extract; and (e) the strong alkali extract. The colloidal ferric hydrate precipitate is transferred to a digestion flask with 20 per cent hydrochloric acid; the dilute alkali extract is neutralized, concentrated under diminished pressure to a small volume, and then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid; the alcoholic filtrate is concentrated under diminished pressure to remove the alcohol, then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid; the filtrate from the extraction with boiling 20 per cent hydrochloric acid and the washings are transferred to a digestion flask with a volume of concentrated hydrochloric acid equal to the volume of the washings; the strong alkali extract is neutralized, concentrated under diminished pressure to a small volume, and then transferred to a digestion flask with an equal volume of concentrated hydrochloric acid. The proteins in these fractions are completely hydrolyzed by boiling for 15 to 20 hours under reflux condensers.

The completely hydrolyzed protein solutions are combined and analyzed according to the Van Slyke method. Some slight modifications, most of which were made necessary by the larger amount of protein material present, have been made. A few other minor changes in the technique have been found advisable in the application of the original method to this type of solution.

Four samples are usually run at the same time. The following chemical methods are used: Van Slyke's method for amino-acid nitrogen and ammonia (amide nitrogen), Plimmer's method (12) for arginine, Denis' modification of Benedict's method for organic sulfur, and the Gunning-Arnold-Dyer modification of the Kjeldahl method for total nitrogen. Duplicate or triplicate determinations were always made whenever possible. The histidine and lysine nitrogen are calculated according to Van Slyke's original method (10)<sup>1</sup>.

<sup>1</sup> The error in Van Slyke's formula for the calculation of histidine nitrogen has been corrected. Instead of

$$\text{Histidine N} = \frac{3}{2} (D - \frac{1}{4} \text{Arg.}) = 1.007D - 1.125 \text{Arg.}$$

we have always used,

$$\text{Histidine N} = \frac{3}{2} (D - \frac{1}{4} \text{Arg.}) = 1.5D - 1.125 \text{Arg.}$$



## DISCUSSION.

Tables I and II give the results of 6 complete analyses of oats, 8 complete analyses of cottonseed meal, 4 of alfalfa, and 6 of corn. In Table I the results are expressed as percentages of total nitrogen in the feed and in Table II as percentages of the feed. The oats contained 1.680 per cent nitrogen, the cottonseed meal 6.796 per cent, the alfalfa 2.628 per cent, and the corn 1.4074 per cent. The weights of feeds taken for each of the various analyses were 60 gm. in the case of oats and corn, 30 gm. in the case of alfalfa, and 15 gm. in the case of cottonseed meal. The oats and corn were ground so as to pass through an 80 mesh sieve, the alfalfa through a 60 mesh sieve, and the cottonseed meal through a 40 mesh sieve.

Although a comparison of the four feeds, from a standpoint of nitrogen distribution, is not intended at this time, attention may be called to a few of the more significant figures, mainly in support of the method. A brief examination of Tables I and II will show that all solutions, residues, precipitates, and other fractions, obtained in the preparation of the hydrolyzed protein solution and in the subsequent Van Slyke analysis of that solution, were analyzed for their nitrogen content. In other words, no fraction, which might in any way contain any portion of the original sample of feed taken for analysis, was discarded without its total nitrogen content having first been determined. This fact should be kept in mind when a comparison of these results is made with those of other workers.

For convenience, our results on the distribution of nitrogen in the feed, are divided into non-protein nitrogen, nitrogen distribution as shown by the Van Slyke analysis, and the nitrogen lost in the method of analysis.

Criticism has been made of the application of the Van Slyke method to the analysis of feeds on the basis of the possible interference of some of the non-protein nitrogenous constituents. In the present method the ether and the alcohol extractions of Eckstein and Grindley (9) are followed by a cold 1.0 per cent trichloroacetic acid extraction. Trichloroacetic acid, being a comparatively strong acid, and also, in dilute solutions, a good protein precipitant, was found to extract the remaining non-protein

nitrogenous substances quite readily, while only a very small amount of protein material was extracted. This small quantity of protein is separated from the non-protein constituents by precipitation with colloidal ferric hydrate<sup>2</sup>.

Although the exact rôle, played in protein metabolism by all of the non-protein nitrogenous constituents of a feed, is at present unknown, it is of interest to note that these four feeds vary markedly in their non-protein nitrogen content. Alfalfa has the highest percentage of non-protein nitrogen with an average of 19.09 per cent, while cottonseed meal has the lowest with an average of 6.20 per cent. Oats and corn have intermediate values of 12.93 and 9.83 per cent, respectively. Judging from these values, which agree very well with those of Grindley and Eckstein (4), roughages have a higher non-protein nitrogen content and concentrates a lower content than the cereals.

Under the heading "nitrogen lost in method of analysis" are found the results of the total nitrogen determinations on the various fractions, the amino-acid content of which escapes analysis by our method of preparing the hydrolyzed protein solution and by the Van Slyke analysis of this hydrolyzed protein solution. The total nitrogen lost is shown in Column U. The nitrogen lost in the preparation of the hydrolyzed protein solution is shown in Column O, representing the nitrogen left in the residue after treatment with the last extracting fluid, 5 per cent NaOH, and in Column P, giving the nitrogen in the alcohol precipitate (starch) of the hot 2 per cent trichloroacetic acid extract. The extraction of starch was not made in the cases of cottonseed meal and alfalfa because of the small amounts present.

The nitrogen lost in the analysis of the hydrolyzed protein solution by the Van Slyke method, consists of (a) the "unadsorbed humin" of Van Slyke (11), filtered from the amyl alcohol-ether aqueous solution during the decomposition of the bases; (b) the nitrogen extracted by the amyl alcohol-ether mixture; (c) the nitrogen in the residue filtered from the solution of the bases; and (d) the nitrogen in the residue filtered from the solution of the filtrate from the bases. As percentages of the total nitrogen of the feed, the total nitrogen lost, as shown in Column U, Table I, is 1.90 per cent in the case of oats, 3.29 per cent in cottonseed meal, 3.85 per cent in corn, and 4.73 per cent in alfalfa.

<sup>2</sup> Dialyzed iron Merck, containing 5 per cent  $\text{Fe}_2\text{O}_3$ .

TABLE I.—*Distribution of the Nitrogen of Oats, Corn, Cottonseed*

	Non-protein nitrogen.				Results of the					
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*
Oats (contain										
	A	B	C	D	E	F	G	H	I	J
	0.655	1.172	11.522	13.319	2.873	2.783	11.016	11.427	0.961	6.504
	0.597	1.163	11.126	12.886	2.968	3.231	11.083	11.652	1.008	5.651
	0.553	1.041	11.766	13.360	3.481	1.498	11.780	11.888	0.976	5.543
	0.555	1.375	11.140	13.070	2.803	1.748	11.566	11.474	0.951	5.942
	0.513	1.307	10.360	12.180	2.611	2.914	11.350	11.892	0.894	5.840
	0.544	1.294	10.860	12.698	3.255	2.926	11.738	11.554	0.876	5.288
Average.....	0.569	1.225	11.129	12.926	3.013	2.516	11.422	11.647	0.944	5.796
Corn† (contains										
	0.050	0.907	9.311	10.358	1.571	1.375	11.729	8.620	1.099	5.245
	0.792	2.341	8.093	11.226	1.796	2.602	12.265	8.868	1.165	5.460
	0.658	2.369	8.395	11.422	1.499	2.284	12.225	8.867	1.186	4.906
	0.240	0.239	6.836	7.315	0.702	2.685	11.241	8.782	0.925	4.748
	0.028	0.305	7.816	8.149	0.758	2.596	12.218	8.762	0.985	3.928
	0.189	1.958	8.356	10.503	1.084	2.278	9.833‡	8.451	1.071	4.702
Average.....	0.326	1.368	8.135	9.829	1.235	2.303	11.936	8.725	1.072	4.832
Cottonseed meal										
	0.021	0.570	4.943	5.534	2.609	3.462	9.455	18.672	0.961	5.486
	0.089	0.618	4.870	5.577	2.609	5.117	9.689	19.050	0.902	6.330
	0.202	0.652	5.053	5.907	2.492	5.459	9.929	18.467	1.068	7.543
	0.109	0.614	5.531	6.254	2.623	4.477	8.892	18.398	1.123	7.240
	0.081	0.506	5.245	5.832	2.981	2.415	9.249	17.520	1.051	8.584
	0.129	0.489	5.722	6.340	2.930	2.650	9.318	19.443	0.948	6.366
	0.081	0.420	6.097	6.598	2.763	2.334	9.002	17.987	0.707	9.351
	0.046	0.506	7.012	7.564	2.772	2.746	9.764	20.102	0.781	6.464
Average.....	0.095	0.547	5.559	6.201	2.722	3.582	9.412	18.705	0.943	7.171

\*Corrected for solubility of the bases.

† The authors are indebted chiefly to Nao Uyei, assistant chemist, for the following

‡ Not included in the average.

§ Determination lost; average result substituted to make up total.

*Meal, and Alfalfa (Expressed as Percentage of Total Nitrogen).*

Van Slyke analysis.			Total non-protein + results of Van Slyke analysis.	Nitrogen lost in method of analysis.							Total.
Lysine N.*	Amino-acid N in filtrate from bases.*	Non-amino-acid N in filtrate from bases.*		N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 3 per cent CCl <sub>4</sub> CO <sub>2</sub> H extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

1.680 per cent N).

K	L	M	N	O	P	Q	R	S	T	U	V
2.182	41.992	4.108	97.195	0.120	0.109	0.558	0.525	0.363	0.024	1.699	98.894
2.821	41.981	2.964	96.245	0.055	0.108	0.835	0.771	0.297	0.033	2.099	98.344
3.445	42.174	3.809	97.954	0.136	0.148	0.566	0.759	0.083	0.015	1.707	99.661
2.386	41.928	4.988	96.946	0.165	0.123	0.851	0.929	0.141	0.025	2.234	99.180
3.426	42.066	3.588	96.761	0.148	0.153	0.340	0.755	0.191	0.020	1.607	98.368
2.787	42.682	3.704	97.503	0.168	0.123	0.833	0.738	0.179	0.033	2.074	99.577
2.841	42.137	3.860	97.100	0.132	0.127	0.664	0.746	0.209	0.025	1.903	99.004

1.4074 per cent N).

2.424	46.090	5.600	94.111	0.505†	0.513	0.930	0.521	0.189	0.065	2.723	96.834
2.418	46.790	0.884†	93.474	0.176	0.163	0.930	0.527	0.206	1.468†	3.470	96.944
2.473	45.059	8.251	98.172	0.147	0.335	1.999	0.515	0.230	0.008	3.234	101.406
1.847	47.750	8.763	94.758	0.107	0.265	3.686	0.372	0.149	0.133	4.712	99.470
2.221	47.962	6.870	94.449	0.113	0.073	2.791	0.531	0.164	0.045	3.717	98.166
1.814	46.574	6.599	92.909	0.137	0.305	5.850	0.418	0.209	0.075	6.994	99.903
2.200	46.704†	7.216	96.052	0.136	0.276	2.698	0.481	0.191	0.065	3.847	99.899

(contains 6.796 per cent N).

4.240	39.981	3.271	93.671	0.220	Extraction not made.	2.274	0.980	0.206	0.036	3.716	97.387
3.060	40.539	3.432	96.305	0.260		1.562	0.906	0.215	0.039	2.982	99.287
3.570	38.852	1.901	95.188	0.302		1.658	0.788	0.220	0.106	3.074	98.262
4.470	39.828	0.161†	93.466	0.233		3.044	1.135	0.245	0.130	4.787	98.253
4.462	41.958	2.681	96.733	0.430§		1.192	0.691	0.428	0.150	2.891	99.624
4.998	41.950	2.290	97.233	0.685		1.019	0.780	0.380	0.096	2.960	100.193
3.600	39.204	3.087	94.633	0.719		0.772	1.026	0.096	0.065	2.678	97.311
5.274	43.481	3.454	101.402	0.589		1.364	1.068	0.133	0.068	3.222	105.624
4.209	40.724	2.874	96.543	0.430		1.611	0.922	0.240	0.086	3.289	99.832

analyses of corn.

TABLE I—

	Non-protein nitrogen.				Result of the					
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*
Alfalfa (contains										
	A	B	C	D	E	F	G	H	I	J
	0.577	1.940	16.466	18.983	3.682	4.483	6.943	7.949	0.924	4.356
	0.577	1.600	16.301	18.478	3.690	3.512	7.104	8.064	1.062	3.655
	0.524	1.988	17.289	19.801	3.597	5.132	8.204	7.523	0.986	3.782
	0.522	1.864	16.712	19.098	3.791	4.796	7.204	8.446	0.991	3.931
Average.....	0.550	1.848	16.692	19.090	3.690	4.481	7.364	7.996	0.991	3.931

Concluded.

Van Slyke analysis.			Nitrogen lost in method of analysis.							Total.	
Lysine N.*	Amino-acid N in filtrate from bases.*	Non-amino-acid N in filtrate from bases.*	Total non-protein + results of Van Slyke analysis.	N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent $\text{CCl}_4$ . $\text{CO}_2\text{H}$ extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

2.628 per cent N).

K	L	M	N	O	P	Q	R	S	T	U	V
4.334	38.349	3.863	93.866	2.663	Extrac-	0.999	0.609	Not de-	0.144	4.415	98.281
4.959	37.681	3.059	91.264	2.335	tion	1.110	0.816	ter	0.639	4.900	96.164
4.008	37.312	1.196	91.541	2.930	not	1.274	0.301	min-	0.604	5.109	96.650
4.434§	38.786	1.927	93.404	2.146	made.	1.261	0.717	ed.	0.377	4.501	97.905
4.434	38.032	2.511	92.520	2.519		1.161	0.611		0.441	4.732	97.252

TABLE II.—*Distribution of the Nitrogen of Oats, Corn, Cotton-*

	Non-protein nitrogen.				Results of Van Slyke						
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*	Lysine N.*
* Oats (contains											
	A	B	C	D	E	F	G	H	I	J	K
	0.0110	0.0197	0.1936	0.2242	0.0482	0.0467	0.1850	0.1919	0.0161	0.1092	0.0366
	0.0100	0.0195	0.1869	0.2164	0.0498	0.0542	0.1862	0.1957	0.0169	0.0949	0.0474
	0.0093	0.0175	0.1977	0.2244	0.0584	0.0251	0.1979	0.1997	0.0164	0.0931	0.0578
	0.0093	0.0231	0.1872	0.2195	0.0486	0.0293	0.1943	0.1927	0.0159	0.0998	0.0400
	0.0086	0.0220	0.1740	0.2046	0.0438	0.0489	0.1906	0.1997	0.0150	0.0981	0.0575
	0.0091	0.0217	0.1824	0.2133	0.0546	0.0491	0.1972	0.1941	0.0147	0.0887	0.0468
Average..	0.0096	0.0206	0.1870	0.2170	0.0506	0.0422	0.1918	0.1956	0.0158	0.0973	0.0477
Corn† (contains											
	0.0007	0.0140	0.1310	0.1458	0.0221	0.0194	0.1651	0.1213	0.0155	0.0738	0.0341
	0.0111	0.0330	0.1139	0.1530	0.0253	0.0366	0.1726	0.1248	0.0164	0.0768	0.0340
	0.0093	0.0333	0.1182	0.1608	0.0211	0.0321	0.1721	0.1248	0.0167	0.0690	0.0348
	0.0034	0.0034	0.0962	0.1030	0.0099	0.0378	0.1582	0.1236	0.0130	0.0668	0.0260
	0.0004	0.0043	0.1100	0.1147	0.0107	0.0365	0.1720	0.1233	0.0139	0.0553	0.0313
	0.0027	0.0276	0.1176	0.1478	0.0153	0.0321	0.1384†	0.1189	0.0151	0.0662	0.0255
Average..	0.0046	0.0193	0.1145	0.1383	0.0174	0.0324	0.1680	0.1228	0.0151	0.0680	0.0310
Cottonseed meal											
	0.0014	0.0387	0.3360	0.3761	0.1773	0.2352	0.6426	1.2689	0.0653	0.3728	0.2882
	0.0061	0.0420	0.3310	0.3790	0.1773	0.3478	0.6585	1.2946	0.0613	0.4302	0.2080
	0.0137	0.0443	0.3434	0.4014	0.1694	0.3710	0.6748	1.2550	0.0726	0.5126	0.2426
	0.0075	0.0417	0.3459	0.4250	0.1783	0.3043	0.6043	1.2503	0.0763	0.4920	0.3038
	0.0055	0.0345	0.3565	0.3963	0.2026	0.1641	0.6286	1.1906	0.0714	0.5834	0.3032
	0.0088	0.0332	0.3889	0.4309	0.1991	0.1801	0.6333	1.3213	0.0644	0.4326	0.3399
	0.0055	0.0285	0.4144	0.4484	0.1878	0.1586	0.6118	1.2224	0.0480	0.6356	0.2447
	0.0032	0.0344	0.4765	0.5140	0.1884	0.1866	0.6635	1.3661	0.0631	0.4393	0.2905
Average..	0.0065	0.0372	0.3778	0.4214	0.1850	0.2434	0.6396	1.2712	0.0641	0.4873	0.2860

\* Corrected for solubility of the bases.

† The authors are indebted chiefly to Nao Uyei, assistant chemist, for the follow-

‡ Not included in the average.

§ Determination lost; average result substituted to make up total.

*seed Meal, and Alfalfa (Expressed as Percentage of Feed).*

analysis.		Total non-protein + results of Van Slyke analysis.	Nitrogen lost in method of analysis.							Total.
Amino-acid N in filtrate from bases*.	Non-amino acid N in filtrate from bases.*		N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent CCl <sub>4</sub> . CO <sub>2</sub> H extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.
1.680 per cent N).										
L	M	N	O	P	Q	R	S	T	U	V
0.7054	0.0690	1.6328	0.0020	0.0018	0.0094	0.0088	0.0061	0.0004	0.0285	1.6614
0.7052	0.0498	1.6169	0.0009	0.0018	0.0140	0.0130	0.0050	0.0006	0.0352	1.6521
0.7085	0.0640	1.6456	0.0023	0.0025	0.0095	0.0128	0.0014	0.0003	0.0286	1.6743
0.7044	0.0838	1.6287	0.0028	0.0021	0.0143	0.0156	0.0024	0.0004	0.0375	1.6662
0.7067	0.0602	1.6255	0.0025	0.0026	0.0057	0.0127	0.0032	0.0003	0.0270	1.6525
0.7071	0.0622	1.6380	0.0028	0.0021	0.0140	0.0124	0.0030	0.0006	0.0348	1.6729
0.7079	0.0648	1.6312	0.0022	0.0021	0.0112	0.0125	0.0035	0.0004	0.0319	1.6632
1.4074 per cent N).										
0.6487	0.0788	1.3245	0.0071†	0.0072	0.0131	0.0073	0.0027	0.0009	0.0383	1.3628
0.6585	0.0124	1.3156	0.0025	0.0023	0.0131	0.0074	0.0029	0.0207	0.0488	1.3644
0.6342	0.1161	1.3817	0.0021	0.0047	0.0281	0.0072	0.0032	0.0001	0.0455	1.4271
0.6720	0.1233	1.3336	0.0015	0.0037	0.0519	0.0052	0.0021	0.0019	0.0663	1.3999
0.6750	0.0967	1.3293	0.0016	0.0010	0.0393	0.0075	0.0023	0.0006	0.0523	1.3816
0.6555	0.0929	1.3076	0.0019	0.0043	0.0823	0.0059	0.0029	0.0011	0.0984	1.4060
0.6573	0.1016	1.3513	0.0019	0.0039	0.0380	0.0068	0.0027	0.0009	0.0541	1.4060
(contains 6.796 per cent N).										
2.7170	0.2223	6.3659	0.0150	Extraction not made.	0.1545	0.0666	0.0140	0.0024	0.2525	6.6184
2.7550	0.2332	6.4548	0.0177		0.1062	0.0616	0.0146	0.0026	0.2027	6.7482
2.6404	0.1292	6.4690	0.0205		0.1127	0.0536	0.0150	0.0072	0.2089	6.6779
2.7067	0.0109‡	6.5319	0.0158		0.2069	0.0771	0.0167	0.0088	0.3253	6.6773
2.8514	0.1822	6.5740	0.0292§		0.0810	0.0470	0.0291	0.0102	0.1965	6.7704
2.8509	0.1556	6.6080	0.0465		0.0693	0.0530	0.0258	0.0065	0.2011	6.8091
2.6643	0.2098	6.4313	0.0489		0.0525	0.0697	0.0065	0.0044	0.1820	6.6132
2.9550	0.2347	6.8913	0.0400		0.0927	0.0726	0.0090	0.0046	0.2190	7.1102
2.7676	0.1953	6.5611	0.0292		0.1095	0.0627	0.0163	0.0058	0.2235	6.7846

ing analyses of corn.



TABLE II—

	Non-protein nitrogen.				Results of Van Slyke						
	Soluble in ether.	Soluble in alcohol.	In filtrate from colloidal iron.	Total non-protein N.	Insoluble humin N.	Soluble humin N.	Acid amide N.	Arginine N.*	Cystine N.*	Histidine N.*	Lysine N.*
Alfalfa (contains											
	A	B	C	D	E	F	G	H	I	J	K
	0.0152	0.0510	0.4327	0.4989	0.0968	0.1178	0.1825	0.2089	0.0243	0.1145	0.1139
	0.0152	0.0420	0.4284	0.4856	0.0970§	0.0923	0.1867	0.2119	0.0279	0.0961	0.1303
	0.0138	0.0522	0.4544	0.5204	0.0945	0.1349	0.2156	0.1977	0.0259	0.0994	0.1053
	0.0137	0.0490	0.4392	0.5019	0.0996	0.1260	0.1893	0.2220	0.0260	0.1033§	0.1165§
Average..	0.0145	0.0486	0.4387	0.5017	0.0970	0.1178	0.1935	0.2101	0.0260	0.1033	0.1165

*Concluded.*

analysis.		Total non-protein + results of Van Slyke analysis.	Nitrogen lost in method of analysis.							Total.
Amino-acid N in filtrate from bases *	Non-amino-acid N in filtrate from bases.*		N in residue after treatment with strong NaOH.	In alcohol precipitate of hot 2 per cent $\text{CO}_2\text{CO}_2\text{H}$ extracts.	Unadsorbed humin (filtered from solution during decomposition of bases).	In amyl alcohol-ether extract.	In residue filtered from solution of bases.	In residue filtered from solution of filtrate from bases.	Total N lost.	Total N accounted for.

2.628 per cent N).

L	M	N	O	P	Q	R	S	T	U	V
1.0078	0.1015	2.4668	0.0700	Extrac-	0.0263	0.0160	Not de-termin- ed.	0.0038	0.1160	2.5828
0.9902	0.0804	2.3984	0.0614	tion	0.0292	0.0214		0.0168	0.1288	2.5272
0.9806	0.0314	2.4057	0.0770	not	0.0335	0.0079		0.0159	0.1343	2.5400
1.0193	0.0506	2.4547	0.0564	made.	0.0331	0.0188		0.0099	0.1183	2.5729
0.9995	0.0660	2.4314	0.0662		0.0305	0.0161		0.0116	0.1244	2.5558

A word of explanation is necessary regarding these fractions. According to Van Slyke's original method (10): "During the distillation [of ammonia] all of the black coloring matter, or melanin, which is formed during the hydrolysis of the proteins, is adsorbed by the undissolved lime." The latter is filtered off, washed, and submitted to Kjeldahl analysis. The results are reported as melanin nitrogen. In the first attempt to apply the Van Slyke method to the analysis of feeds, Grindley, Joseph, and Slater (1), used the original method directly. The results for their melanin nitrogen included, therefore, any nitrogenous substances in the insoluble residue of the feed as well as the melanin or humin *formed* during the hydrolysis. Nollau (2) filtered off the insoluble residue remaining after the hydrolysis of the feed and determined by the Van Slyke method "the amino-acid content of certain commercial feedingstuffs and other sources of protein" in the filtrate. Attention was called to this fact and to the introduction of certain errors by this procedure by Grindley and Slater (3) in a second paper on "the quantitative determination of amino-acids of feedingstuff by the Van Slyke method." The nitrogen, heretofore called melanin nitrogen by them and also by Van Slyke, was called humin nitrogen in this paper.

The humin nitrogen is divided by Gortner (7) into "acid-insoluble" and "acid-soluble" (absorbed by lime) humin, the sum of the two being the total humin nitrogen. Gortner and Holm (8) report the two fractions under separate headings as insoluble humin nitrogen and soluble humin nitrogen. Other workers have reported this humin nitrogen fraction under such various headings as "humin N absorbed by lime" (Osborne, Van Slyke, Leavenworth, and Vinograd, 13), "humin nitrogen adsorbed by magnesia" (Miller, 14), and as "melanin nitrogen" divided into "a b c fraction" and "lime fraction" (Neidig and Snyder, 15). In the present paper the expressions insoluble and soluble humin have been retained. The humin nitrogen of Table III is the sum of the insoluble and the soluble humin nitrogen. It is possible that the nitrogen, or parts of it at least, in the fractions listed in Columns Q, R, S, and T of Tables I and II properly belongs to the total humin nitrogen figures but for the present it will not be included.

Here again the terminology is confusing. In Van Slyke's improved method (11) of decomposing the basic phosphotungstates by the amyl alcohol-ether method, the following statement is made:

"In some cases the aqueous and ether-amyl alcohol layers do not separate readily with a clean boundary between them. This effect is due to the presence of a slight amount of humin which may have escaped previous adsorption by calcium hydrate. In this case the unadsorbed humin is carried down with the basic phosphotungstates, and fouls the solution when their precipitate is decomposed as above described [amyl alcohol-ether method]. In order to clear the solution up, it is all, without separation of the aqueous and ether-amyl alcohol layers, passed through a Buchner funnel with suction."

Osborne, Van Slyke, Leavenworth, and Vinograd (13) in one case purified their basic phosphotungstates by reprecipitation with phosphotungstic acid and state that "all the coloring matter which accompanied the bases was extracted by the amyl alcohol and ether." The nitrogen taken up by the organic solvents is determined and reported by them as "humin N in amyl alcohol extract." Gortner and Holm (8) separated, besides the "acid-insoluble humin" and the "acid-soluble humin," a "phosphotungstic humin." This was determined by submitting the barium phosphotungstate precipitate to Kjeldahl analysis.<sup>3</sup> This fraction of humin nitrogen is undoubtedly the "humin N in amyl alcohol extract" of Osborne, Van Slyke, Leavenworth, and Vinograd and the "unadsorbed humin" of Van Slyke. This fraction is again divided by Miller (14) into "humin N insoluble in amyl alcohol" and "humin N in amyl alcohol extract."

Neidig and Snyder (15) found that a dark-colored substance usually formed along with the bases when the latter were precipitated with phosphotungstic acid. These authors refer to this substance as the "phosphotungstic humin" of Gortner and Holm. Until the present method was adopted, this dark-colored substance, which was sometimes of a sticky nature, was found repeatedly in this laboratory. With this substance present it was almost impossible to wash the basic phosphotungstates thoroughly. By the present method, outlined above, the basic

<sup>3</sup> These authors used Van Slyke's original barium hydroxide method of liberating the bases.

phosphotungstates of all four of the feeds reported here were white, gray, or slightly cream-colored granular precipitates free from any dark-colored material. However, during the decomposition of the phosphotungstates by the amyl alcohol-ether method, a scum of a dark color is formed making filtration necessary. This residue is washed carefully with ammonia-free water, amyl alcohol, and ether, and submitted to Kjeldahl analysis. The results are reported in Column Q under the heading "unadsorbed humin," the name used by Van Slyke. The clear amyl alcohol-ether extract of the phosphotungstic acid is also submitted to Kjeldahl analysis and the nitrogen content reported in Column R as the nitrogen in amyl alcohol-ether extract.

On concentrating the solution of the bases a small gray residue has been found to settle out. Van Slyke, in the original method of freeing the bases by means of barium hydrate, stated that this residue was barium phosphotungstate, which was to be filtered off and discarded. This residue has always been encountered in this laboratory even when the amyl alcohol-ether method of decomposing the basic phosphotungstates was employed. In the method used in the present work this residue is filtered off, washed carefully, and its total nitrogen determined. The results are shown in Column S. After making the filtrate from the bases up to volume (200 cc.) a residue similar to the one in the solution of the bases separates out. This is usually very small in amount, but since it has been found to be present in all cases, the nitrogen has been determined (Column T).

No attempt has been made to determine the nature of the nitrogenous constituents in these fractions. The total nitrogen has been determined in them entirely for the purpose of showing the accuracy or inaccuracy of the method as applied to the analysis of feeds.

As percentages of the total nitrogen of the feed, the humin nitrogen of oats is 5.53 per cent, of corn 3.54 per cent, of cottonseed meal 6.30 per cent, and of alfalfa 7.36 per cent.

The completeness with which the nitrogen is extracted from the finely ground feeds is shown in Column O, Table I, which gives the percentages of the total nitrogen of the feeds remaining in the residues after the last extraction with 5 per cent NaOH solution. The nitrogen in the residue from the oats, as shown by the average,

is, 0.132 per cent of the original amount present; from the corn, 0.136 per cent; from the cottonseed meal, 0.430 per cent; and from the alfalfa, 2.519 per cent. Osborne and Mendel (16) found 6.0 per cent of the total nitrogen of whole corn left after extractions with 10 per cent KCl solution, 90 per cent alcohol, and 0.2 per cent KOH solution. Miller (14) in a study of the distribution of nitrogen in the alfalfa seed makes a 0.5 per cent KOH extraction and leaves between 9 and 10 per cent of the total nitrogen in the residue.

The nitrogen distribution as shown by the Van Slyke analysis includes the insoluble humin nitrogen, the soluble humin nitrogen, acid amide nitrogen, arginine nitrogen, cystine nitrogen, histidine nitrogen, lysine nitrogen, amino-acid nitrogen in the filtrate from the bases, and the non-amino-acid nitrogen in the filtrate from the basis. The question has been raised in several papers on the analysis of the proteins of feeds by the Van Slyke method that the various fractions ordinarily designated "arginine nitrogen", "histidine nitrogen," etc., may not be accurately described by these terms, on account of the heterogeneous nature of the nitrogenous constituents as well as other substances present. In the total absence of experimental evidence on this point, it seems fair to assume that the above terms are as properly applied to the fractions of nitrogen obtained in the analysis of feeds by the above described method as to the corresponding fractions obtained in the analysis of pure proteins.

It is of interest to note the variation in the basic nitrogen of the different feeds, since we know more of the requirements of animals for basic amino-acids than for any other group. The arginine nitrogen varies from about 8 per cent of the total nitrogen in alfalfa to 18.7 per cent in cottonseed meal. The cystine nitrogen is about the same in all cases but these results may, perhaps, be a little low. The histidine nitrogen is lowest in alfalfa with a value of 3.9 per cent of the total nitrogen and is highest in cottonseed meal with a value of 7.2 per cent. Lysine nitrogen is lowest in corn with a value of 2.2 per cent, slightly higher in oats with 2.8 per cent, and highest in alfalfa with 4.43 per cent. The total basic nitrogen as percentage of total nitrogen is 31.03 per cent in cottonseed meal, 21.23 per cent in oats, 17.35 per cent in alfalfa, and 16.83 per cent in corn.

In Table III the average results obtained in Table I are compared with the results on the same feeds obtained by Grindley and associates reported by Grindley (6) and by Nollau (2).

As a whole, the determinations of the different investigators do not agree well, although in some instances the agreement is quite satisfactory. The results obtained by the authors of this paper agree with those of Grindley and associates slightly better than they do with those of Nollau. The lack of concordant results is due, chiefly, to differences in methods used. Grindley and associates hydrolyzed the finely ground feeds, determined the acid amide nitrogen in the hydrolysate, filtered off the humin nitrogen, and proceeded to analyze the filtrate according to the Van Slyke method. Their results were based on the total nitrogen in the feed. Nollau removed the fat by extracting the finely ground feed with ether. The samples were then completely hydrolyzed and the insoluble humin filtered off. The total nitrogen determined in the filtrate was the basis for calculation of results of the nitrogen distribution as obtained by the Van Slyke procedure. By the improved method described above the non-protein nitrogenous constituents, most of the carbohydrates, and the fiber are removed before hydrolysis. The objectionable features of the previous methods are thereby obviated completely or at least greatly reduced. Considering the differences in procedure, Nollau's results for humin nitrogen should be lower than the results for the humin nitrogen of Grindley and associates. In general this is true. Nollau's procedure should also lead to correspondingly higher results for the remaining nitrogen values, considered on the basis of the total nitrogen of the feed. With a few exceptions this is found to be true, but the results are usually higher than this difference of procedure would warrant. The lower results for ammonia nitrogen, the amino-acid nitrogen, and non-amino-acid nitrogen in the filtrate from the bases of the method used in this work are expected because of the removal of the non-protein nitrogen. There are some differences in the results that cannot be explained on the basis of differences of procedure. For example, Nollau reports no lysine in oats while we find 2.84 per cent, he reports 8.53 per cent of lysine in corn while we find only 2.2 per cent, and again he reports no non-amino-acid nitrogen in the filtrate from the bases in corn

TABLE III.  
*Comparison of Results with Those of Previous Investigators (Results Expressed as Percentage of Total Nitrogen).*

	Oats.		Nollau.		Corn.		Nollau.		Cottonseed meal		Alfalfa.	
	Hamilton, Nevens, and Grindley.	Grindley and associates, 1915.	Grindley and associates, 1915.	Nollau.	Hamilton, Nevens, and Grindley.	Grindley and associates, 1915.	Nollau.	Hamilton, Nevens, and Grindley.	Grindley and associates, 1915.	Nollau.	Hamilton, Nevens, and Grindley.	Grindley and associates, 1915.
Ammonia N.....	11.42	13.06	13.31		11.94	12.53	4.63	9.41	10.45	14.06	8.17	8.44
Humin N.....	5.53	9.94	2.97		3.54	9.77	7.00	6.30	7.78	6.27	7.36	15.79
Arginine N.....	11.65	11.42	11.42		8.73	8.49	16.19	18.71	19.52	12.77	8.00	7.68
Cystine N.....	0.94	1.16	4.48		1.07	2.68	4.06	0.94	0.65	2.74	0.99	0.88
Histidine N.....	5.80	4.32	9.58		4.83	3.50	4.45	7.17	5.47	7.57	3.93	7.44
Lysine N.....	2.84	3.49	0.00		2.20	1.17	8.53	4.21	4.78	1.94	4.43	4.10
Amino-acid N in filtrate from bases.....	42.14	51.72	43.49		46.70	52.26	49.69	40.72	42.82	45.02	38.03	44.02
Non-amino-acid N in filtrate from bases.....	3.86	7.90	11.29		7.22	11.17	0.00	2.87	5.43	7.49	2.51	9.79
Ether-soluble N.....	0.57				0.33			0.10			0.55	
Alcohol-soluble N.....	1.22				1.37			0.55			1.85	
Non-protein N soluble in 1 per cent $\text{CCl}_4\text{CO}_2\text{H}$ -in filtrate from colloidal Fe.....	11.13				8.14			5.56			16.69	
N lost in method of analysis.....	1.90				3.85			3.29			4.73	
Total.....	99.00	103.01	96.54	94.55	99.92	101.57	97.86	99.83	96.90	97.86	97.24	98.14



while we find 7.22 per cent. The results Nollau obtained for cystine are in all cases much higher than those obtained by us.

Miller (14) in his study of the distributon of nitrogen in the alfalfa seed precipitates the protein from a 0.5 per cent KOH extract with acetic acid and analyzes the precipitate which contains only 60 per cent of the total nitrogen of the seed, according to the Van Slyke analysis. The results obtained, therefore, cannot be considered as representing the distribution of nitrogen in the entire seed. Dowell and Menaul (17) report on the "nitrogen distribution of the proteins extracted by dilute alkali from pecans, peanuts, kafir, and alfalfa." Their method, which was similar to that used by Miller, was in case of alfalfa, as follows:

"Alfalfa which was ground to pass a 40 mesh sieve was extracted with a 0.3 per cent sodium hydroxide, and 62 per cent of the nitrogen compounds were extracted. 61 per cent of the nitrogen extracted was precipitated when the solution was made slightly acid with acetic acid. The purity of the precipitated protein was found to be 85 per cent, using the factor 6.25."

Our results on alfalfa are compared with those of Dowell and Menaul, in Table IV. The agreement is obviously very poor.

TABLE IV.  
*Nitrogen Distribution of Alfalfa.*

	Dowell and Menaul.*	Hamilton, Nevens, and Grindley.†
NH <sub>3</sub> -N.....	6.8	8.17
Humin N.....	7.8	7.36
Arginine N.....	11.01	8.00
Histidine N.....	6.26	3.93
Cystine N.....	0.85	0.99
Lysine N.....	5.26	4.43
Monoamino N.....	53.53	38.03
Non-amino N.....	8.48	2.51

\* Average of two analyses; results expressed as percentage of total nitrogen of the protein preparation.

† Average of four analyses; results expressed as percentage of total nitrogen of the alfalfa.

Dowell and Menaul make the statement: "We have taken advantage of the fact that all proteins are soluble in basic solu-

tion to separate them from the other substances in foods and feeds which make it impracticable to apply the Van Slyke method to determine the nitrogen distribution." While the precipitated proteins in the case of pecans and peanuts represented a slightly larger percentage of the total nitrogen of the foodstuff, the proteins precipitated from the alkali extract of alfalfa represented less than 40 per cent of the total nitrogen of the alfalfa. Evidently a considerable fraction of the proteins of these vegetable materials is *not* soluble in basic solvents. Yet these authors conclude that "the extractions of the proteins with dilute alkaline solutions may enable us to obtain the amino-acid composition of foods and feeds by means of the Van Slyke method." With such large percentages of the nitrogen of a food or feed remaining in the unanalyzed residue, the significance of the results obtained on the extracted proteins may be seriously questioned and we doubt whether the proteins of feeds may be quantitatively separated from the other constituents by any such simple procedure as this.

#### SUMMARY AND CONCLUSIONS.

1. The amino-acid contents of oats, corn, cottonseed meal, and alfalfa, as determined by the Van Slyke method, are reported in this paper.

2. The objectionable parts of previous procedures for the application of the Van Slyke method to the determination of amino-acids of feeds have been obviated completely or at least greatly reduced by the following features of the methods used in this work: (a) The non-protein nitrogenous constituents are removed by extractions with absolute ether, cold absolute alcohol, and cold 1.0 per cent trichloroacetic acid; (b) the starch is removed by a hot 2.0 per cent trichloroacetic acid extraction; and (c) the fiber is not present during the hydrolysis of the proteins.

3. The Van Slyke method for the determination of the chemical groups characteristic of the amino-acids of proteins can be applied to the quantitative estimation of the amino-acids of feeds.

4. By further application of available methods for the estimation of other amino-acids to hydrolyzed protein solutions, prepared in a manner similar to that described for this work, it may be possible to obtain further important knowledge concerning the nutritive value of the proteins of foods and feeds.

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The quantitative estimation of the amino-acids of the proteins of other common feeds is in progress at this laboratory. A study of the nitrogen distribution of the non-protein nitrogenous fraction as well as that of the humin fractions is under consideration.

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# THE SYNTHESIS OF INACTIVE PARA- AND ANTI-HYDROXYASPARTIC ACIDS (AMINOMALIC ACIDS).

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The increasing recognition of the importance of the hydroxy-amino-acids in protein chemistry is an incentive to their more complete investigation. The particular case of hydroxyaspartic acid or aminomalic acid is of interest for several reasons. It represents the lower homologue of  $\beta$ -hydroxyglutamic acid already identified as a constituent of various proteins, and hence itself may not improbably be found among the proteins. Indeed Skraup (1) in 1904 stated that he had actually found hydroxyaspartic acid among the products of hydrolysis of casein. A careful perusal of Skraup's description of the supposedly new amino-acid does not lead to much confidence in the accuracy of the deductions drawn. In the first place the experiments are described in a fashion which makes repetition impossible; secondly no evidence is adduced that the substance in question was a dibasic acid, neither are molecular weight, optical rotation, nor a complete analysis recorded. The substance is stated to be sparingly soluble in cold water, and melts at 305–320°. A copper salt of supposedly normal constitution containing 3.5 to 4 molecules of water of crystallization, was analyzed for carbon and hydrogen, while a single nitrogen determination was made on 86 mg. of the free acid. Without further analytical or chemical evidence Skraup concludes, "Es liegt demnach bestimmt die Oxyamidobernsteinsäure vor, die bisher überhaupt noch nicht beschreiben worden ist." Experiments on the synthesis of the acid were stated to be in progress but have not been reported.

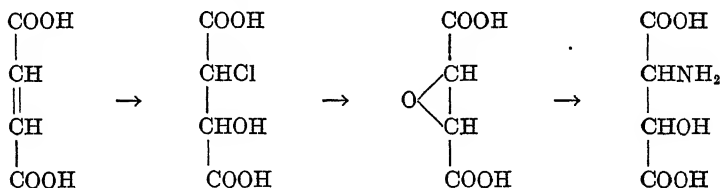
In the same year Erlenmeyer (2) wrote, "Wie im experimentellen Theile gezeigt werden wird, ist es mir gelungen . . . von Oxalylhippursäureester aus zur Amidoäpfelsäure, welche

kurzlich von Skraup als Spaltungsprodukt des Caseins nachgewiesen wurde, zu gelangen." But a careful examination of the experimental part of this and other papers by Erlenmeyer has failed to disclose any further reference to the acid.

Almost simultaneously Neuberg and Silbermann (3) announced another synthesis of the acid by the limited action of nitrous acid upon mesodiaminosuccinic acid. A small amount of a readily soluble copper salt (1.2 gm.) was obtained which apparently contained no water of crystallization and which gave an analysis agreeing excellently with that calculated for the normal salt. The free acid was not obtained in sufficient amount for analysis but its melting point is given as  $314-318^{\circ}$ , in substantial agreement with Skraup's observation. As a matter of fact, as will appear in the experimental portion of the paper, hydroxyaspartic acid does not give a soluble copper salt under the conditions employed by Neuberg and Silbermann; namely, boiling with copper carbonate. Almost the whole of the acid remains as a light blue very sparingly soluble copper salt, containing water of crystallization, and the salt has an abnormal composition containing three equivalents of copper. Furthermore, the acid does not melt at the temperature given by either Skraup or by Neuberg and Silbermann but begins to decompose slowly above about  $230^{\circ}$  but is not melted even at  $360^{\circ}$ . The writer believes that Neuberg and Silbermann's compound contained no hydroxyaspartic acid and the apparent correspondence of the analysis of their copper salt with that calculated for the normal compound and the agreement of the melting point with Skraup's observation remains unexplained.

On the other hand experiments made on a small preliminary scale by Lossen (4) in 1906, while described by the author as incomplete, indicated that an acid,  $C_2H_2 \cdot (OH \cdot NH_2)(COOH)_2 + H_2O$ , presumably an aminomalic acid, was formed by the action of ammonia on fumarylglucidic acid. Neither the free acid nor barium salt described by Lossen agree in detail with the products obtained by the writer, but there is no question that much aminomalic or hydroxyaspartic acid was present in Lossen's substances. In the experimental part of the paper details are given showing clearly the difficulty of satisfactorily crystallizing the products from the above reaction.

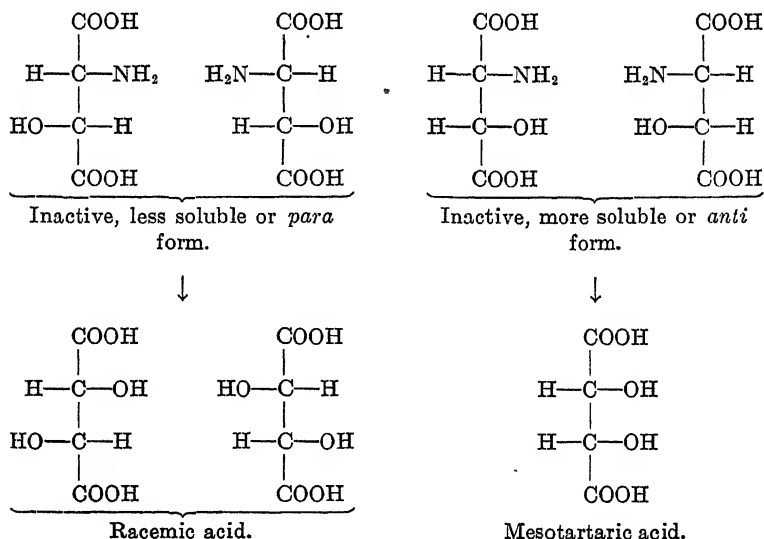
The synthesis made use of in the present investigation is based on the action of ammonia on chloromalic acid, prepared by the action of chlorine on sodium fumarate. The action of ammonia on chloromalic acid first results in the production of fumaryl-glycidic acid so that the reaction is essentially similar to that used by Lossen. The changes may be represented as follows:



Chloromalic acid reacts in the cold with strong aqueous ammonia but even after 2 weeks the conversion into hydroxyaspartic acid is incomplete and the products are difficult to separate. On the other hand hydroxyaspartic acid is not completely stable in aqueous solution even at 125° and in alkaline or acid solution it is still more readily decomposed. On the whole it was found that the reaction was best carried out by heating the chloromalic acid with five parts of concentrated aqueous ammonia for about 10 hours in an iron autoclave immersed in a boiling water bath. On isolating the amino-acid as described in the experimental part of this paper, or by many other methods which need not be described, the product was obtained in the form of a viscous mass which became friable on treatment with glacial acetic acid or with alcohol and which was extraordinarily soluble in water. The purified product analyzed satisfactorily for  $\text{C}_4\text{H}_7\text{O}_5\text{N}$  and its molecular weight as determined by Barger's method or by titration was close to 149. The whole of the nitrogen was in the amino form and could be liberated with nitrous acid. Early efforts at crystallization failed, but eventually it was found possible to obtain well formed crystals which separated slowly from aqueous solutions of moderate concentration. Once in possession of crystals for seeding purposes it was found very easy to crystallize new preparations. On successive fractional crystallization it was found possible to resolve the substance into two isomeric forms with similar chemical properties but differing in solubility and crystalline form. The least soluble form which

is present in smaller amount than the other is very sparingly soluble in cold water when pure. On decomposition with nitrous acid it gives chiefly, if not exclusively, racemic acid. The more soluble form crystallizes slowly and has a great tendency to form supersaturated solutions, but even this isomer requires about thirty parts of cold water to dissolve it. The effect on the solubility of the one form caused by the presence of the other is remarkable. The more soluble form on treatment with nitrous acid gives mesotartaric acid. The two forms are interconvertible to a certain extent and in particular the less soluble form is produced on heating a 25 per cent aqueous solution of its isomer for several hours at 120–125°.

From the preceding facts it is clear that two optically inactive stereoisomeric forms of hydroxyaspartic acid exist corresponding in structure to mesotartaric and racemic acids. Each of these two forms should be separable into active components giving a total of four active and two inactive forms. The resolution into active components is as yet incomplete, but will be reported shortly. The various isomeric forms of hydroxyaspartic acid in relation to mesotartaric and racemic acids are shown in the subjoined formulas:



Hydroxyaspartic acid thus furnishes a good example of the occurrence of two inactive resolvable isomers of a compound containing two dissimilar asymmetric carbon atoms. The example gains in interest partly from the fact that hydroxyaspartic acid represents the simplest known case of this type of isomerism and also on account of the direct relationship with the classical isomerism of the tartaric acids.

The separate designation of the two inactive hydroxyaspartic acids offers considerable difficulties such as are often encountered in other asymmetrical derivatives of succinic acid. The use of *cis* and *trans* for differentiating such saturated compounds is generally agreed to be undesirable. The use of the term "*fumaroid*" for the less soluble and higher melting isomer and "*maleinoid*" for the more soluble and lower melting one seems inappropriate in the present case although the fumaroid form of hydroxyaspartic acid would be the one related to mesotartaric acid and as is well known fumaric acid is directly oxidizable to racemic and not mesotartaric acid. The use of the term "*allo*" for a second isomer is convenient in cases such as isoleucine when one form can be appropriately assigned the simpler name without a prefix. On the whole the use of the terms "*para*" and "*anti*" already made use of by Bischoff for isomeric alkylsuccinic acids appears least open to objection. In the present case the differentiation is not made on the basis of assigning the "*para*" prefix to the higher melting isomer since neither form possesses a definite melting point. Rather, the term "*para*" is assigned to the less soluble form which is convertible into *para*-tartaric or racemic acid while the more soluble form is designated "*anti*" since it gives *anti*- or mesotartaric acid on replacement of its amino group by hydroxyl.

The *anti* and *para* forms of hydroxyaspartic acid are readily differentiated by the unaided eye after a little experience and still more readily under the microscope. It is hoped to obtain a good crystallographic description of the two forms. Both forms give an intense pyrrole reaction on gentle heating. Most of the salts are similar though the acid calcium salts show differences which are to be referred to later. The phenylhydantoin derivative of the *anti* acid is much more readily soluble than the *para* compound. Reference must be made to the copper and zinc salts



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both of which are of abnormal composition. Both *para*- and *anti*-hydroxyaspartic acids, when dissolved in two equivalents of sodium hydroxide, give sparingly soluble precipitates with neutral copper or zinc acetates, the reaction of the solution becoming acid. Analyses of the salts show the presence of three equivalents of metal, indicating that the hydrogen of the hydroxyl group has been replaced by metal. A similar phenomenon is well known to occur with tartrates and malates and Fischer and Leuchs (5) has found that the copper salt of isoserine is similarly abnormal. The relationship of isoserine and hydroxyaspartic acid is obviously a close one.



Isoserine.

Salt contains 2 equivalents of Cu.

Hydroxyaspartic acid.

Salts contain 3 equivalents of Cu  
or Zn.

Hydroxyaspartic acid prevents the precipitation of iron and copper by excess of alkali as is the case with the analogous tartrates and malates.

Finally a word may be said in regard to the possible presence of hydroxyaspartic acid in proteins. The present situation appears to be that the acid certainly has not yet been identified and Skraup's assertions to the contrary are undoubtedly ill founded. On the other hand, certain newer experiments made by the writer have given negative results, but it is not at all certain that the methods used were adequate. The problem is a difficult one and needs further investigation.

Experiments on the preparation of hydroxyaspartic acid ethyl esters, using ethyl alcohol and hydrogen chloride, indicated their formation in good yield. The esters were liberated from the hydrochlorides by means of potassium carbonate and extracted with ether. On attempting to distil the esters under moderately low pressure (10 mm.), a great deal of decomposition occurred and only very little impure ester, boiling at about

140°, was obtained. It appears improbable that the ester method would be useful for the detection of hydroxyaspartic acid in proteins.

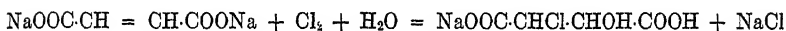
Hydroxyaspartic acid in both *anti* and *para* forms reduces potassium permanganate slowly in acid, neutral, or alkaline solution. On oxidation of the neutral salts with sodium hypochlorite or chloramine-T, a good deal of glyoxal is formed together with some tartronic semialdehyde. Neither form gives Fenton's reaction for tartaric acid with hydrogen peroxide and ferrous sulfate, though they give various color reactions with phenols and sulfuric acid resembling more or less those given by malic and tartaric acids.

On heating the *anti* form with strong aqueous potash it was completely decomposed, and on heating with a little hydrochloric acid at 125° it was also found to be unstable. On heating it with water at 125° for 4 hours a partial conversion into the *para* form was accomplished. The reverse change could also be demonstrated though less readily; namely, the formation of the *anti* from the *para* acid.

#### EXPERIMENTAL PART.

*Chloromalic Acid.*—The following modification of Lossen's method has proved useful for the preparation of considerable quantities of chloromalic acid. A large bottle (10 to 20 liters) of approximately known capacity is filled with water and inverted in a trough. Chlorine gas from a cylinder of liquid chlorine is rapidly passed in until the bottle is filled when it is removed and corked. The weight of chlorine in the bottle is then calculated. An amount of fumaric or maleic acid equivalent to the chlorine is then weighed out, suspended in water, and neutralized with sodium hydroxide, using phenolphthalein as indicator. The sodium fumarate is diluted with ice water to about 1 per cent concentration and a few cc. are rapidly poured into the bottle of chlorine which is then closed with a stopper carrying a rubber tube dipping into the bulk of the fumarate solution. On agitating the gas bottle, absorption of the chlorine rapidly occurs and the whole of the solution is readily sucked over. The mixture is allowed to stand until the following morning when it

is neutralized with sodium hydroxide, an amount of alkali being required which is exactly half of that used originally to neutralize the fumaric acid.



A 20 liter gas bottle serves for quantities of fumaric acid in the neighborhood of 110 gm.; its use will be found far more convenient than the use of strong chlorine water described by Lossen. Crystallized barium chloride ( $2\frac{1}{2}$  equivalents) is then added to the neutralized chloromalate solution and the whole vigorously shaken for half an hour on a machine so as to obtain a readily filterable precipitate. After a few hours standing the barium chloromalate is filtered off, washed with water, and dried carefully in a warm place as it retains water mechanically with unusual persistence. The air-dry barium salt is crushed, weighed, and then transferred to a thick walled bottle and covered with ether. Concentrated hydrochloric acid is then added by degrees with shaking and cooling until about a 10 per cent excess has been added. The ether layer is decanted from the aqueous barium chloride suspension and the extraction repeated with fresh ether four or five times. The combined ether extracts are washed with a few drops of water and then evaporated at a low temperature. Chloromalic acid remains as an oil which quickly solidifies to an opaque crystalline mass of the pure substance. The yield averages 45 to 50 per cent of the theoretical amount calculated from the fumaric acid used.

*Fumarylglcydic Acid.*—The filtrate from the barium chloromalate still contains a good deal of dissolved salt and this may be converted into the less soluble barium salt of fumarylglcydic acid by adding an additional equivalent of sodium hydroxide and allowing the mixture to stand for a further 24 hours. The barium salt thus obtained as described by Lossen is filtered off and dried. Instead of using Lossen's method of liberating the free acid which has not given good results in the writer's hands it is better to decompose the salt at  $0^\circ$  with concentrated hydrochloric acid in a calculated amount as already described for barium chloromalate, but using ethyl acetate as solvent instead of ether. The ethyl acetate is removed by distillation under reduced pressure

when fumarylglucidic acid readily crystallizes out in amount equivalent to 20 to 25 per cent of the fumaric acid originally employed. Fumarylglucidic acid thus obtained serves equally well as chloromalic acid for the preparation of hydroxyaspartic acid.

*Hydroxyaspartic Acids.*—Chloromalic acid (56 gm.) is added by degrees to 300 cc. of concentrated aqueous ammonia contained in a porcelain beaker surrounded by an ice bath. A little ether may be added with advantage to promote the dissolution of the acid. Ammonia gas is then passed in rapidly until the mixture approaches saturation. The covered beaker is then placed in an iron autoclave suspended in a water bath which is brought to the boiling point for about 10 hours. If the reaction is carried out in iron tubes without other protection a good deal of iron remains dissolved in the clear solution and is decidedly difficult to remove quantitatively. The use of the porcelain beaker is therefore strongly recommended. After heating, the contents of the beaker are diluted with an equal volume of water and slightly more than two equivalents (28 gm. instead of 26.6 gm.) of sodium hydroxide are added. The solution is then evaporated to a small volume under reduced pressure so as to remove most of the ammonia. The residue is taken up in water and made fairly strongly acid to Congo red with nitric acid. Silver nitrate is then added in slight excess over that needed to remove the whole of the chloride. The filtrate is diluted to about 750 cc. and a concentrated solution of 150 gm. crystallized lead acetate is added. The solution is then incompletely neutralized by the addition with stirring of either dilute sodium hydroxide or ammonia leaving the supernatant fluid still distinctly acid with the liberated acetic acid. The lead precipitate is thoroughly washed with cold water, transferred to a flask, suspended in water, and decomposed with hydrogen sulfide under slight pressure in the customary fashion. After complete decomposition the mixture is heated on the water bath, and filtered from lead sulfide, and concentrated under diminished pressure. A syrup is finally obtained which may be conveniently purified by stirring it successively with glacial acetic acid and methyl alcohol. A white sticky mass results which after washing with alcohol quickly becomes brittle and dusty in the desiccator. The product represents almost

entirely a mixture of the two forms of hydroxyaspartic acid, as is shown by the following analyses. The substance was dried *in vacuo* over phosphorus pentoxide at 60°.

Prep. I.	0.1180 gm. substance:	0.1400 gm. CO <sub>2</sub> ,	0.0499 gm. H <sub>2</sub> O.
• Prep. II.	0.2132 " "	:0.2541 " CO <sub>2</sub> ,	0.0950 " H <sub>2</sub> O.
	0.2052 " "	:0.0191 " N (Kjeldahl).	
	0.0106 " "	:1.76 cc. N, 18°, 754 mm. (Van Slyke).	
C <sub>4</sub> H <sub>7</sub> O <sub>5</sub> N.	Calculated.	C 32.2,	H 4.7, N 9.4.
	Found.	C 32.3, 32.7,	H 4.7, 4.95, N 9.31, 9.46.

On titration 1.850 gm. required 12.7 cc. normal sodium hydroxide to neutralize, using litmus paper as indicator. This is equivalent to a molecular weight of 146, compared with a calculated value of 149. On employing Barger's micro method for molecular weight determinations it was found that an aqueous solution of the acid containing 2.78 per cent was in equilibrium with an 0.18 normal solution of tartaric acid indicating a molecular weight of 154. Silver and barium salts on analysis also gave results in close accord with the preceding figures.

With regard to the separation of hydroxyaspartic acids it may be mentioned that the silver or mercury salts may be used to replace the use of lead, but they offer no significant advantage. The product is also readily precipitated by excess of barium hydroxide as the neutral barium salt but curiously enough it is almost impossible in the writer's experience to recover the acid satisfactorily by decomposition with sulfuric acid since a point is soon reached when addition of either barium hydroxide or sulfuric acid or both fails to produce a precipitate of barium sulfate and the product is heavily contaminated with inorganic substances.

*Separation of Para- and Anti-Hydroxyaspartic Acids.*—Thus far simple fractional crystallization from water is the only method employed for the separation of the two isomers, although it is possible that the differences in solubility of the acid calcium salts may eventually furnish a more convenient method. It is a curious fact that while the *para* and *anti* forms are relatively sparingly soluble when once separated, the mixture of acids separated as described in the preceding section retains its extreme solubility in water apparently indefinitely. In order to separate the two acids it is convenient to dissolve the mixed product in

about five parts of hot water and on cooling, either expose to the air or better, seed the mixture with a few crystals of the *para* acid obtained from a previous operation. Crystallization is slow, but on standing over night a fair crop of crystals usually accumulates, chiefly made up of the *para* form. The *para* form is easily identified by the fact that its crystals are usually small, white, and opaque, somewhat resembling aspartic acid, whereas the *anti* form crystallizes into large stout crystals, often a couple of centimeters long, which are transparent while moist and only become opaque on drying. Under the microscope the *para* acid appears to crystallize in small cubes or plates while the *anti* form crystallizes in hexagonal plates and thick prisms of complicated form.

The first crop of crystals containing excess of the *anti* form is most conveniently purified by washing it with a little warm water in which the *anti* acid dissolves much more quickly, and then recrystallizing the residue once or twice from boiling water until it appears perfectly homogeneous.

The *anti* form crystallizes slowly from the first mother liquor and care should be taken that the solution should not become too concentrated. The crystals are best removed from time to time and finally the accumulated product should be rapidly dissolved in hot water, rejecting any sparingly soluble and slowly dissolving *para* acid which may be identified with the microscope. Crystallization is allowed to take place slowly at room temperature. The yield of *para* acid seldom exceeds 20 per cent of the mixed product while the amount of *anti* form varies considerably but always greatly exceeds the other. In well conducted experiments as much as 60 to 70 per cent of the crude product may be obtained in the *anti* form while often much remains in the mother liquors in a difficultly recoverable condition. Occasionally a product is encountered, crystallizing in well formed needles and giving at first the appearance of homogeneity, but on slow recrystallization from water it will be found to be a mixture of the two forms already dissolved.

*Para-Hydroxyaspartic Acid*.—The purified acid, separated as described in the preceding section, was recrystallized from water and obtained in the form of small opaque cubic crystals. It has no sharp melting point but on heating it slowly decomposes above

235° and gives a solid mass which does not melt even at 350°. On ignition it gives vapors which furnish a strong pyrrole reaction with the pine-splinter test. The acid dissolves in about 300 parts of cold water, but in the presence of isomerides or other impurities its apparent solubility is enormously increased.

Analysis. 0.1941 gm. substance: 0.2278 gm. CO<sub>2</sub>, 0.0872 gm. H<sub>2</sub>O.  
 0.0155 " " : 2.58 cc. N, 20°, 756 mm. (Van Slyke).  
 C<sub>4</sub>H<sub>7</sub>O<sub>5</sub>N. Calculated. C 32.2, H 4.70, N 9.40.  
 Found. C 32.0, H 4.99, N 9.48.

*Phenylhydantoin Derivative of Para-Hydroxyaspartic Acid.*—1 gm. of the acid was dissolved in 15 cc. normal sodium hydroxide and an excess of phenylisocyanate (1 gm.) was added by degrees with frequent shaking. After the cyanate had been completely decomposed the mixture was filtered from diphenylurea and the filtrate acidified with concentrated hydrochloric acid (3 cc.). No separation occurred, so the solution was evaporated on the water bath to small bulk. A separation of the hydantoin occurred at this stage, but as it is apt to be contaminated with sodium chloride it was convenient to separate the bulk of the latter by taking the hydantoin up in methyl alcohol, filtering, and concentrating. On cooling the hydantoin quickly separated out and was spread on porous tile and then recrystallized from 50 per cent methyl alcohol in which it is freely soluble. It crystallizes in bundles of fine white needles and melts sharply at 201.5–202.5° (uncorrected). The substance while freely soluble in water is less soluble in water, alcohol, and acetone than the corresponding derivative of the *anti* acid. It is very slightly soluble in chloroform.

Analysis. 0.1500 gm. substance: 0.2894 gm. CO<sub>2</sub>, 0.0564 gm. H<sub>2</sub>O.  
 C<sub>11</sub>H<sub>16</sub>N<sub>2</sub>O<sub>6</sub>. Calculated. C 53.1, H 4.00.  
 Found. C 52.6, H 4.18.

The salts of the two acids in most particulars resemble each other closely as regards properties and composition. The salts of the alkali metals and of magnesium are freely soluble in water while those of the heavy metals are all sparingly soluble. One noteworthy difference in the salts of the two acids is the fact that the acid calcium and barium salts of the *para* acid are decidedly

less soluble than those of the *anti* acid and unlike the latter do not melt in boiling water.

*Acid Calcium Salt of Para-Hydroxyaspartic Acid.*—The salt was obtained by adding clear lime water until a suspension of the *para* acid in hot water was just neutral to litmus. On concentrating slightly, fine glistening quadrilateral plates readily separate. The same salt is obtained by adding calcium acetate to a hot saturated solution of the acid. Occasionally the crystals appear as coffin-shaped plates. The substance is highly characteristic and the air-dried salt contains 5 molecules of water of crystallization which are given off at  $120^{\circ}$  *in vacuo*.

Analysis. 0.1295 gm. substance:0.0281 gm.  $\text{H}_2\text{O}$ , 0.0415 gm.  $\text{CaSO}_4$ .

$(\text{C}_4\text{H}_5\text{O}_5\text{N})_2\text{Ca}\cdot 5\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  21.1, Ca 9.37.

Found.  $\text{H}_2\text{O}$  21.7, Ca 9.44.

*Neutral Calcium Salt (Para).*—On dissolving one equivalent of the acid in two equivalents of ammonia or sodium hydroxide solution, and then adding calcium acetate, the neutral calcium salt is obtained as a white sparingly soluble granular precipitate. It contains some water of crystallization which is removed at  $120^{\circ}$  *in vacuo*. The dry salt was found to contain 21.3 per cent calcium, the calculated value being 21.4.

*Acid Barium Salt (Para).*—This salt was prepared in the same fashion as the acid calcium salt by adding barium hydroxide to a hot solution of the acid. It is important to have the final reaction of the solution very slightly acid to litmus as otherwise the salt is contaminated with some of the sparingly soluble neutral salt. The acid barium salt is moderately soluble even in cold water and readily soluble in hot water. When it separates rapidly it forms a granular powder, but when crystallized slowly it gives well formed plates, containing 3 molecules of water of crystallization which it loses completely at  $135^{\circ}$  *in vacuo*.

Analysis. 0.3025 gm. substance:0.0235 gm.  $\text{H}_2\text{O}$ , 0.1415 gm.  $\text{BaSO}_4$ .

$(\text{C}_4\text{H}_5\text{O}_5\text{N})_2\text{Ba}\cdot 3\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  11.1, Ba 28.1.

Found.  $\text{H}_2\text{O}$  11.1, Ba 27.5.

*Neutral Barium Salt (Para).*—The salt is most easily obtained by adding a hot aqueous solution of the acid to an excess of barium hydroxide solution. An immediate precipitation of a



very insoluble barium salt takes place and the precipitate is at once filtered off, washed with water, and dried. It appears to be stable and anhydrous.

Analysis. 0.2618 gm. substance:0.2156 gm. BaSO<sub>4</sub>.  
                   (C<sub>4</sub>H<sub>4</sub>O<sub>5</sub>N)Ba. Calculated. Ba 48.2.  
   Found. Ba 48.4.

*Copper Salt (Para).*—The only copper salt obtained in a pure condition is the very sparingly soluble compound which contains three equivalents of copper to one of the acid and hence is an abnormal salt. It is best obtained by neutralizing the *para* acid with two equivalents of sodium hydroxide and then adding a moderate excess of neutral copper acetate. The reaction of the medium turns acid and a voluminous light blue powder is precipitated. The product is soluble in excess of alkali giving a product resembling Fehling's solution and is also soluble in a large excess of copper acetate. The composition of the air-dried product varies slightly but most closely accords with the following formula: (C<sub>4</sub>H<sub>4</sub>O<sub>5</sub>N)<sub>2</sub>Cu<sub>3</sub>·8H<sub>2</sub>O. The exact determination of the combined water is somewhat difficult and is best carried out under greatly diminished pressure over phosphorus pentoxide at a temperature of about 135°. The extremes and average of a number of analyses are given below:

(C <sub>4</sub> H <sub>4</sub> O <sub>5</sub> N) <sub>2</sub> Cu <sub>3</sub> ·8H <sub>2</sub> O. Calculated.	H <sub>2</sub> O 22.9, Cu 30.4, N 4.47, C 15.3.
Found.	H <sub>2</sub> O 20.5–24.2, Average 22.7.
	Cu 29.6–31.1, Average 30.3, N 4.48.
	C 15.6.

*Zinc Salt (Para).*—The zinc salt was obtained in precisely the same way as that employed for the copper salt, substituting zinc acetate for copper acetate. The reaction of the solution becomes acid and the preprecipitated white sparingly soluble salt contains three equivalents of zinc to one of acid. The analysis indicates (C<sub>4</sub>H<sub>4</sub>O<sub>5</sub>N)<sub>2</sub>Zn<sub>3</sub>·7H<sub>2</sub>O as the most probable formula for the air-dried salt. The combined water was determined by drying over phosphorus pentoxide at 2 mm. pressure at 125°.

Analysis. 0.2165 gm. substance:0.0432 gm. H<sub>2</sub>O, 0.0862 gm. ZnO.  
                   0.0210 " " :1.70 cc. N, 21°, 760 mm. (Van Slyke).  
                   (C<sub>4</sub>H<sub>4</sub>NO<sub>5</sub>)<sub>2</sub>Zn<sub>3</sub>·7H<sub>2</sub>O. Calculated. H<sub>2</sub>O 20.5, Zn 31.9, N 4.56.  
   Found. H<sub>2</sub>O 20.0, Zn 32.1, N 4.58.

*Silver Lead and Mercury Salts (Para).*—Most\* of the heavy metals produce sparingly soluble precipitates with hydroxyaspartic acid or its salts. The composition of the lead and mercury salts varies according to the conditions of their formation, from the acid salts, to neutral and basic salts. Some of these have been analyzed but it is not easy to secure perfectly uniform products. The silver salt on the other hand has a constant normal composition. It is obtained as a white curdy insoluble precipitate on adding silver nitrate to *para*-hydroxyaspartic acid, neutralized with two equivalents of ammonia or sodium hydroxide. It is not particularly sensitive to light.

Analysis. 0.2920 gm. substance:0.1747 gm. Ag.

$C_4H_5O_5NaAg_2$ . Calculated. Ag 59.5.

Found. Ag 59.8.

*Anti-Hydroxyaspartic Acid.*—The more soluble acid, separated as previously described, was recrystallized repeatedly from water. It is freely soluble in hot water but requires about thirty-three parts of cold water (18°) for solution. As already stated its apparent solubility is enormously increased by the presence of its isomer or other impurity and it shows considerable tendency to the formation of supersaturated solutions.

Analysis. 0.1501 gm. substance:0.1761 gm.  $CO_2$ , 0.0607 gm.  $H_2O$ .

0.0150 " " :2.54 cc. N, 20°, 756 mm. (Van Slyke).

$C_4H_7O_5N$ . Calculated. C 32.2, H 4.70, N 9.40.

Found. C 32.0, H 4.50, N 9.58.

The molecular weight determined by titration, using litmus paper as indicator, was found to be 147, compared with a calculated value of 149 (0.300 gm. required 10.2 cc. of 0.2 N sodium hydroxide). Using Barger's capillary tube method a 2.622 per cent solution was found equivalent to 0.18 normal tartaric acid, giving a value of 146 for molecular weight.

*Phenylhydantoin Derivative of Anti-Hydroxyaspartic Acid.*—The reaction was carried out as described for the corresponding *para* compound. The methyl alcohol extract crystallized rather less readily and was too soluble to permit of convenient crystallization from water or alcohol. Crystallization was most readily effected by dissolving the compound in hot acetone in which

it is easily soluble and then adding two volumes of chloroform. The hydantoin crystallizes readily in nacreous plates suggestive of leucine in appearance. It melts sharply at 196–198° uncorrected, and is sparingly soluble in chloroform or ethyl acetate.

Analysis. 0.1825 gm. substance: 0.3545 gm. CO<sub>2</sub>, 0.0721 gm. H<sub>2</sub>O.  
 $C_{11}H_{10}N_2O_5$ . Calculated. C 52.8, H 4.00.  
 Found. C 52.9, H 4.30.

The following salts of *anti*-hydroxyaspartic acid were all prepared as described for those of the *para* acid, hence only the analyses and any points of difference between the two series are recorded below.

*Acid Calcium Salt (Anti)*.—The salt differs from the *para* compound in that it melts to a gum in boiling water. It is freely soluble and is best obtained by evaporation of its aqueous solution at room temperature in a desiccator. It contains 4 molecules of water of crystallization, and easily passes over into the neutral salt.

Analysis. 0.1514 gm. substance: 0.0260 gm. H<sub>2</sub>O, 0.0395 gm. CaCO<sub>3</sub>.  
 0.0150 “ “ : 1.65 cc. N, 21°, 760 mm. (Van Slyke).  
 $(C_4H_5NO_5)_2Ca \cdot 4H_2O$ . Calculated. H<sub>2</sub>O 17.7, Ca 9.80, N 6.30.  
 Found. H<sub>2</sub>O 17.2, Ca 9.80, N 6.23.

*Neutral Calcium Salt (Anti)*.—The salt is a white granular sparingly soluble substance containing close to 2 molecules of water of crystallization.

Analysis. 0.1521 gm. substance: 0.0243 gm. H<sub>2</sub>O, 0.0677 gm. CaCO<sub>3</sub>.  
 $C_4H_5N_2O_5Ca \cdot 2H_2O$ . Calculated. H<sub>2</sub>O 16.1, Ca 18.0.  
 Found. H<sub>2</sub>O 16.0, Ca 17.8.

*Acid Barium Salt (Anti)*.—Melts with some difficulty in boiling water and separates from cool solutions in crystalline nodules. It dissolves in about twenty-five parts of cold water and contains 3 molecules of water of crystallization which are removed at 105°.

Analysis. 0.2383 gm. substance: 0.0278 gm. H<sub>2</sub>O, 0.1097 gm. BaSO<sub>4</sub>.  
 $(C_4H_5O_5N)_2Ba \cdot 3H_2O$ . Calculated. H<sub>2</sub>O 11.1, Ba 28.1.  
 Found. H<sub>2</sub>O 11.6, Ba 27.1.

*Neutral Barium Salt (Anti).*—A very sparingly soluble granular powder containing no water of crystallization, closely resembling the *para* salts.

Analysis. 0.1606 gm. substance:0.1307 gm.  $\text{BaSO}_4$ .  
 $(\text{C}_4\text{H}_5\text{O}_5\text{N})\text{Ba}$ . Calculated. Ba 48.2.  
 Found. Ba 48.1.

*Copper Salt (Anti).*—In appearance and properties as well as composition the copper salt of the *anti* acid closely resembles that of the *para* acid.

Analysis. 0.1788 gm. substance:0.0393 gm.  $\text{H}_2\text{O}$ , 0.0679 gm.  $\text{CuO}$ .  
 0.0300 “ “ :2.32 cc. N,  $20^\circ$ , 762 mm. (Van Slyke).  
 $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Cu}\cdot 8\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  22.9, Cu 30.4, N 4.47  
 Found.  $\text{H}_2\text{O}$  22.0, Cu 30.4, N 4.31

*Zinc Salt (Anti).*—This salt closely resembles the *para* salt. It is sparingly soluble and contains three equivalents of zinc to one of the acid.

Analysis. 0.1984 gm. substance:0.0397 gm.  $\text{H}_2\text{O}$ , 0.0782 gm.  $\text{ZnO}$ .  
 $(\text{C}_4\text{H}_4\text{O}_5\text{N})_2\text{Zn}_3\cdot 7\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  20.5, Zn 31.9.  
 Found.  $\text{H}_2\text{O}$  20.0, Zn 31.7.

*Silver and Lead Salts (Anti).*—The silver salt was obtained as a white insoluble curdy precipitate containing 59.7 per cent of silver (calculated 59.8). The normal lead salt is obtained as a dense white heavy precipitate on adding lead acetate to a neutral solution of the sodium or ammonium salt of the *anti* acid. It contained no water of crystallization and gave on analysis 58.1 per cent of lead (calculated 58.3). On adding lead acetate to a solution of the free acid a microcrystalline insoluble salt of complex composition (48.3 per cent lead) separates out, and on washing with water is slowly converted into the normal salt.

*Action of Nitrous Acid on Anti- and Para-Hydroxyaspartic Acids.*—In each case the *anti* or *para* acid (1.49 gm.) was dissolved in 150 cc. of water and 3 cc. of concentrated hydrochloric acid. The solutions were kept at room temperature and sodium nitrite (1 gm.) was added by degrees in the course of a day. Nitrogen was freely evolved on agitating the solutions. The following day the solutions were almost neutralized with ammonia

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using litmus as indicator, and an excess of calcium acetate was added. The precipitated calcium salts which separated rather slowly were in each case filtered off, dissolved in a few drops of hydrochloric acid, and reprecipitated with ammonia. The *anti*-hydroxyaspartic acid gave a granular calcium salt, composed of small prisms and contained close to 3 molecules of water of crystallization, which it lost at 170° and appeared identical in every respect with calcium mesotartrate prepared for comparison.

Analysis. 0.1463 gm. substance:0.0331 gm. H<sub>2</sub>O, 0.0789 gm. CaSO<sub>4</sub>.  
 $C_4H_4O_6Ca \cdot 3H_2O$ . Calculated. H<sub>2</sub>O 22.3, Ca 16.5.  
 Found. H<sub>2</sub>O 22.5, Ca 16.0.

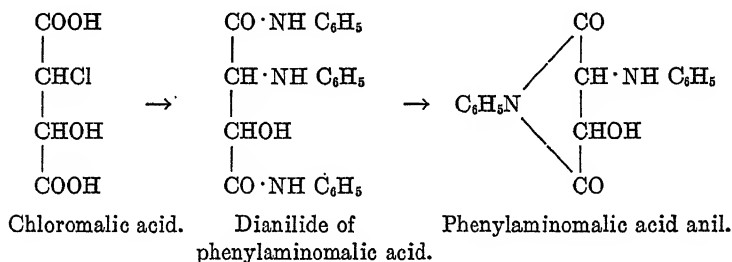
The *para*-hydroxyaspartic acid gave a calcium salt which was slightly more soluble than that from the *anti* acid and was made up of needles. Few if any of the mesotartrate prisms were found on microscopic examination. The salt contained close to 4 molecules of water which were removed at 170° and appeared identical with calcium racemate prepared for comparison.

Analysis. 0.1838 gm. substance:0.0498 gm. H<sub>2</sub>O, 0.0989 gm. CaSO<sub>4</sub>.  
 $C_4H_4O_6Ca \cdot 4H_2O$ . Calculated. H<sub>2</sub>O 27.7, Ca 15.4.  
 Found. H<sub>2</sub>O 27.1, Ca 15.8.

The yield of mesotartaric and racemic acids was only equivalent to about 25 per cent of the theoretical amount.

*Action of Aniline on Chloromalic Acid.*—In connection with the study of other bases than ammonia upon chloromalic acid, the following two derivatives were obtained by the action of aniline. Chloromalic acid (1 mol) with aniline (4 mols) were heated for 3 hours in a flask placed in a paraffin bath at 130°. The sticky mass was well washed with dilute hydrochloric acid to remove excess of aniline and then heated with alcohol. A small amount of sparingly soluble substance which proved to be an "anil" derivative of phenylaminomalic acid was filtered off and subsequently recrystallized from glacial acetic acid in which it is readily soluble when hot but sparingly soluble at room temperature. It crystallizes in bright yellow plates and melts at 238–239° (uncorrected). From the alcoholic filtrate a dianilide of phenylaminomalic acid was obtained which was recrystallized from 90 per cent methyl alcohol. It crystallizes in nodular clumps

of bright yellow needles and on heating softens above  $200^{\circ}$  and melts at  $210\text{--}211^{\circ}$  (uncorrected). The yield of the latter substance is considerably greater than that of the "anil". Its reaction may be represented as follows:



Analyses. 0.1102 gm. anilide: 0.2850 gm.  $\text{CO}_2$ , 0.0543 gm.  $\text{H}_2\text{O}$

0.1000 " " : 0.0112 " N (Kjeldahl).

$\text{C}_{22}\text{H}_{21}\text{O}_3\text{N}_3$ . Calculated. C 70.7, H 5.58, N 11.2.

Found. C 70.6, H 5.50, N 11.2.

0.1169 gm. anil: 0.2901 gm.  $\text{CO}_2$ , 0.0537 gm.  $\text{H}_2\text{O}$ .

$\text{C}_{16}\text{H}_{14}\text{O}_3\text{N}_2$ . Calculated. C 68.0, H 5.0.

Found. C 67.7, H 5.10.

The hydrolysis of the preceding derivatives with formation of phenylaminomalic acid has not yet been satisfactorily accomplished.

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## STUDIES IN INORGANIC BLOOD PHOSPHATE.

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As preliminary to a series of experiments to be noted in the latter part of this paper, a number of inorganic phosphate determinations in the whole blood of rabbits under normal and experimental conditions has been made. These covered first the inorganic phosphate of the blood of normal rabbits fed on the usual laboratory diet of hay, bread, and oats; and second, the curve of inorganic blood phosphate following the intravenous injection of phosphate solutions.

### *Normal Bloods.*

No series of observations on the inorganic phosphate of the blood of normal rabbits has been found in the literature. Iversen's (1) observations on the "acid-soluble" phosphate in normal bloods are prefaced by remarks on his technique in which he states that the investigated bloods (rabbit, guinea pig, rat, cat, etc.) showed 5 to 7 mg. of phosphorus as inorganic phosphate per 100 cc. of blood, whereas in his experimental reports the total "acid-soluble" phosphate is given us about 30 mg. of P per 100 cc. of blood.

The method of Bell and Doisy (2) for the estimation of inorganic blood phosphate was used under the personal direction of Doctor Doisy to whom acknowledgment is gratefully given. The method, a recent one, is simple and as judged by a number of duplicate determinations, accurate. Occasionally a sample of blood was met with in which the color reaction of phosphomolybdic acid failed to appear. Possibly the quantity of ammonium



molybdate added was insufficient to combine with the phosphate in the usual manner. We have often noted a turbidity which is not phosphomolybdate when the ammonium molybdate is added. This undoubtedly indicates another insoluble compound of molybdic acid with the probable failure of the formation of ammonium phosphomolybdate—an essential upon which the determination hinges. It has been found that these anomalous results can be corrected by doubling the quantity of ammonium molybdate added; *i.e.*, 1 cc. of 10 per cent ammonium molybdate in 2 N  $\text{H}_2\text{SO}_4$ .

Oxalated blood was obtained by nicking the marginal ear vein and collecting in amounts of a little over 2 cc. by allowing it to run into a calibrated test-tube containing a few crystals of potassium oxalate. From this tube 2 cc. were pipetted off for estimation. The potassium oxalate was tested for phosphate and was found free from this impurity.

The results of twenty-six readings of normal rabbit blood were as follows:

Mg. per 100 cc. of blood.			
4.1	5.8	4.5	4.4
4.2	6.0	5.3	4.5
4.5	5.1	7.1	3.9
5.4	5.4	6.8	4.5
4.3	5.0	5.7	2.6
5.3	4.6	4.5	4.2
	4.7	4.3	
Average. . . . . 4.87 mg. of P as inorganic phosphate per 100 cc. of blood.			

The striking fact in these figures is the uniform level of this substance in the normal blood. All but three of these readings lie between 4 and 6 mg. of P per 100 cc. of blood. The average is seen to be 4.87 mg. The high reading is 7.1 mg. and the low, 2.6 mg. Eleven of these determinations were made in duplicate.

Several of the determinations were carried out on each of three different rabbits over periods up to  $50\frac{3}{4}$  hours.

Rabbit No.	Date.	Time.	Mg. of P per 100 cc. of blood.
1	Oct. 11	1.30 p. m.	4.1
		2.30 "	4.2
		3.30 "	4.5
	Oct. 12	10.15 a. m.	5.4
		3.00 p. m.	4.3
	Oct. 13	10.15 a. m.	5.3
2	Oct. 12	11.15 a. m.	5.8
		2.00 p. m.	6.0
		3.50 "	5.1
	Oct. 13	10.15 a. m.	5.4
	" 14	2.00 p. m.	5.0
3	Oct. 14	2.00 p. m.	4.6
		3.30 "	4.7
	Oct. 15	3.00 "	4.5

Here again a striking constancy of inorganic phosphate level is shown.

#### *Experimental Injection of Phosphate Solutions.*

The literature of this field of the present study offers a greater number of previous reports, largely because of interest in the phosphorus-calcium balance in the blood and its relation to tetany. The most important work in this connection is that of Binger (3), who plotted a curve of toxicity of injected orthophosphates varying with their pH. He found that in dogs as much as 250 mg. of P per kilo of body weight in the form of orthophosphoric acid could be injected intravenously without development of tetany, whereas 200 mg. in the form of  $\text{Na}_2\text{HPO}_4$  and 150 mg. in the form of  $\text{Na}_3\text{PO}_4$  each caused tetany. He further plotted a curve of serum phosphorus, using Marriott and Haessler's method of estimation, following injection. This curve shows the same prompt fall to normal as the present experiments show. He presents also the inverse curve of calcium that blood analyses in various forms of tetany indicate. Iversen (1) found in the rabbit a similar curve to those about to be reported. His injections were made over a prolonged period. In a later paper (4) he found that both *in vitro* and *in vivo* the red corpuscles take

up gradually a proportion of the acid-soluble phosphate present in the plasma. *In vivo* this means that following the injection of phosphates, the curve of acid-soluble phosphate in the cells falls more slowly than that in the plasma.

All of the present series of experiments were done in the same manner with the exception of the solutions and amounts used. The rabbit, on the usual diet, was weighed and a sample of blood was taken for estimation of inorganic phosphorus as described in the first portion of this paper. The solution at room temperature was then injected as rapidly as possible by gravity from a burette into the marginal vein of the ear not previously employed for the procuring of a blood sample. This injection took about 5 to 8 minutes on the average. Observations for toxic effects were made and blood samples were withdrawn at varying intervals. The solutions used were: (1) M/15  $\text{NaH}_2\text{PO}_4$ ; and (2) a mixture of  $\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  so calculated as to present a solution of approximately pH 7.3 and approximately isotonic with the blood.

The curves (Figs. 1 to 8) present the results of the estimations following the injection in 8 of the below 10 experiments.

Experiment No.	Solution 1.	Solution 2.
	cc.	cc.
4	100	
5	75	
6	75	
7	75	
8	75	
9	75	
10	50	
11	50	
19		75
20		75

Experiments 4 and 9 present no curves on account of technical errors in collecting the blood samples. They are included in this report because the former succumbed with positive symptoms of tetany and the latter was the only animal to die without symptoms of tetany.

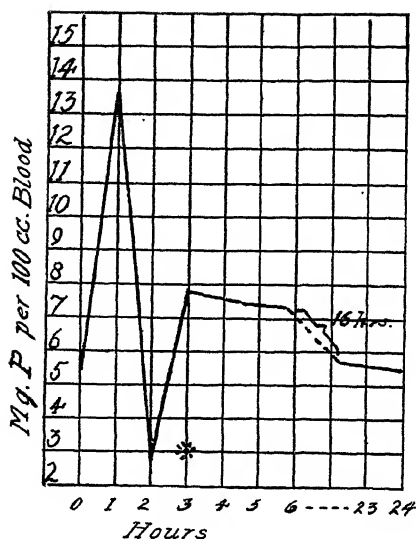


FIG. 1. Experiment 5. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms. The reading marked \* is probably erroneous. The color reaction resembled that mentioned in the text as occasionally disturbing the estimations.

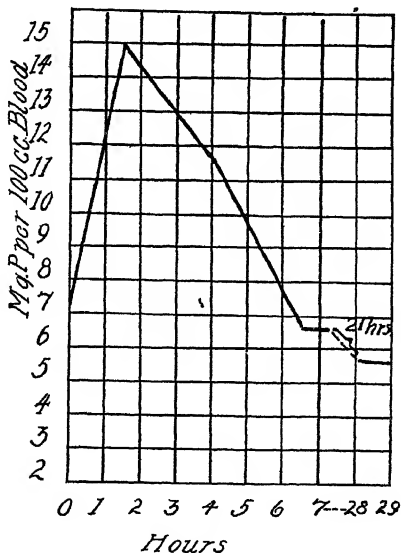


FIG. 2. Experiment 6. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Salivation; cyanosis. Survived.

The protocols of Experiments 15 and 18 have been omitted on account of the occurrence of the phenomenon mentioned in the discussion of the Bell and Doisy method. This obviously inval-

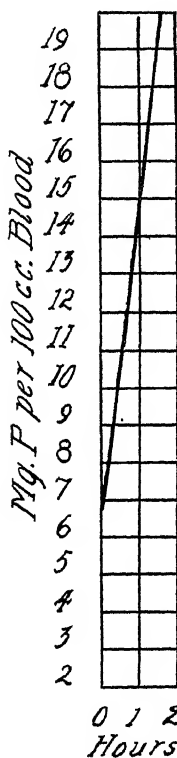


FIG. 3.

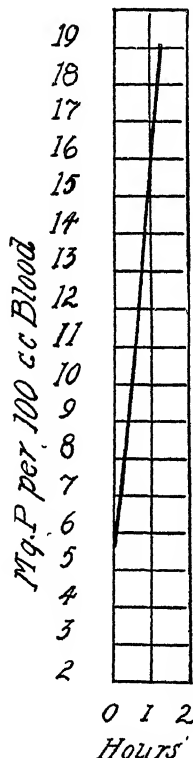


FIG. 4.

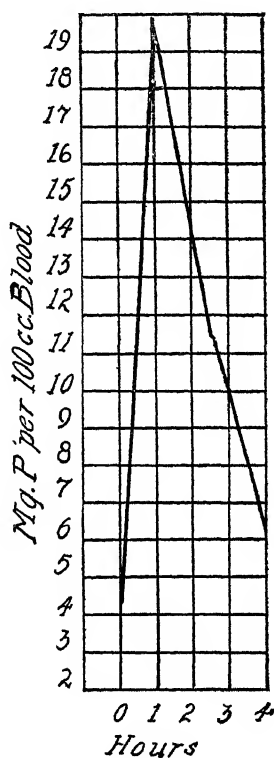


FIG. 5.

FIG. 3. Experiment 7. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Tetany; salivation. Dead in about 3 hours.

FIG. 4. Experiment 8. 75 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. Tetany; salivation. Dead in 18 hours.

FIG. 5. Experiment 10. 50 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms.

idated the results which showed in the former instance a late rise of blood phosphate and in the latter, an experiment on a dog, no rise at all.

There is in these curves a demonstration of the strong tendency of the rabbit's body to maintain the constant level of inorganic blood phosphate found in the estimations on normal rabbits given above. The rapidity, with which concentrations of blood

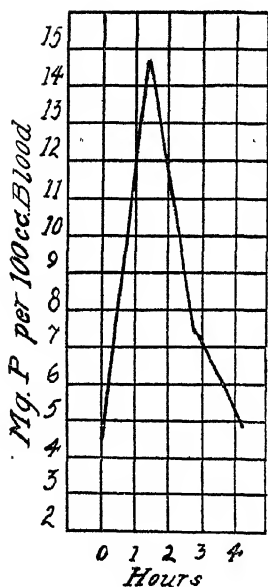


FIG. 6.

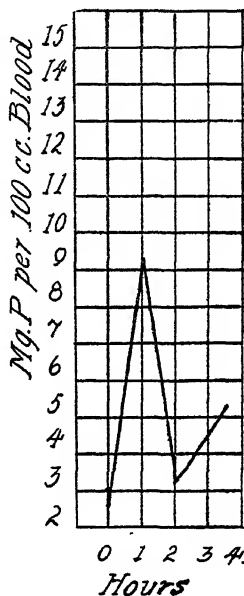


FIG. 7.

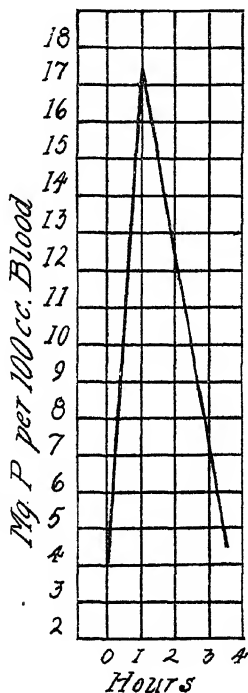


FIG. 8.

FIG. 6. Experiment 11. 50 mg. of P per kilo— $\text{NaH}_2\text{PO}_4$  solution. No symptoms.

FIG. 7. Experiment 19. 75 mg. of P per kilo—mixed phosphate solution. No symptoms.

FIG. 8. Experiment 20. 75 mg. of P per kilo—mixed phosphate solution. Slight tetanic symptoms. Survived.

phosphate four to five times the normal disappear, is extreme. In this connection it must be remembered that the first post injection estimation is made at about 1 hour. Previous to the end of this hour the phosphate concentration must be enormously

higher. Within 4 hours after injection the normal level has been reached. No attempt was made to follow the blood calcium. Somewhat similar experiments on the calcium content of the whole blood of rabbits are reported by Clark (5), with similar results. It is clear that the phosphate-calcium balance is maintained by the organism at a constant level, and that any deviation from this level will be promptly corrected.

The symptoms observed following the injection of massive doses of phosphates deserve mention on account of the interest in the toxicity of these substances. When 100 mg. of P per kilo of  $\text{NaH}_2\text{PO}_4$  were injected (Experiment 4) there was the development of tremors followed by distinct tetanic convulsions and death in 3 hours. These convulsions could be brought on in the rigid extensors of the leg by handling or attempting to flex. Similar observations were made in Experiments 7 and 8 with death in 3 and 18 hours respectively. These two rabbits received 75 mg. of P per kilo of the same substance. Rabbit 9 which also received the last named dosage, died about 12 hours after injection without symptoms of tetany. The only symptoms noted were cyanosis and some form of circulatory collapse which prevented the free flow of blood from the vein. One rabbit (Experiment 20) which received 75 mg. of P per kilo of the mixed phosphate solution showed dyspnea and a questionable spasm of the back and neck muscles. There were no convulsions. An interesting symptom which has for lack of a better name been noted in the curves as "salivation" occurred in several instances. This consisted in a free flow of thin mucoid material from the mouth in the first 2 to 3 hours following injection. Whether or not this represents simply an attempt on the part of the organism to dispose of the excess fluid injected is not clear. No estimation of phosphate in this secretion was made. Nor were controls with the injection of salt solution carried out.

#### *Cod Liver Oil Feeding.*

In connection with recent clinical and experimental reports (6, 7, 8) on the calcification of bone in rickets following the feeding of cod liver oil, it was thought that possibly some light might be thrown on the phenomenon by a similar series of experiments, substituting cod liver oil *per os* for the injection of phosphates. These experiments were entirely negative.

The same general method was employed. Cod liver oil was given by stomach tube in 20 cc. amounts and the inorganic blood phosphate was followed at somewhat longer intervals to allow of absorption. In three such experiments in the rabbit and one in the dog no alteration of inorganic blood phosphate was observed.

### *Blood Phosphate and the Calcification of Callus.*

The purpose for which the above experiments were carried out was to form a groundwork on which a surgical problem with biochemical aspects could be carried out. As this work presented negative results it will be but briefly mentioned here.

Provided that the periosteal cell reaction about a fractured bone is normally active, the length of time during which the patient is incapacitated depends primarily on the rate of deposition of calcium salts in the soft callus. With this idea as a basis many experimental and clinical attempts have been made to increase the speed of this calcification.

The present attempt has been based on the following facts. If one adds *in vitro* a solution of phosphates to a solution containing calcium and corresponding in composition to the inorganic composition of the blood, there is precipitated a calcium compound corresponding to the composition of bone (9). Furthermore, the injection of phosphates intravenously results in an immediate fall in the calcium content of the blood, which stays low until the excess phosphate disappears (3). This latter fact suggested the possibility that the reaction definitely known to occur *in vitro* might also occur *in vivo*. The fate of the calcium which disappears from the blood stream is unknown, but it seemed reasonable to assume that it might be directed to a site where there is under ordinary circumstances a tendency for calcium to be deposited. Such a site is, of course, furnished by a soft callus.

The experiments were therefore conducted on the following lines: A bone defect was made with a saw and after varying intervals allowing for the periosteal reaction, the phosphate content of the blood was increased by intravenous injection. The calcification of the fracture was then followed by the x-ray in both the experimental and control animals.



The rabbits were taken in pairs from the same litter and the fore legs x-rayed. A transverse segment of bone about 3 mm. in thickness was then removed from the ulna of each rabbit with the Albee saw, with care to disturb the remaining periosteum as little as possible. Ether anesthesia was used and the wound closed with silk. At the end of an interval varying from 7 to 20 days, the rabbits were again x-rayed. The experimental rabbit then received an injection in the marginal ear vein of a m/15 solution of acid sodium phosphate in the proportion of 50 mg. of phosphorus per kilo of body weight. None of the rabbits showed signs of distress nor was there evidence of tetany. X-rays were taken within the next 6 to 8 hours and again in most instances at later intervals. Eleven pairs of rabbits were subjected to this type of experiment without infection of the wounds. One experiment was conducted by feeding the rabbit cod liver oil instead of injecting phosphate. The results of the experiments showed in no case an undoubted increase of the speed of calcification in the injected rabbits. Two of the above eleven experiments were conducted with the addition of the intraperitoneal injection of a vital stain, sodium alizarin sulfonate (10), as a measure of calcium deposit but in neither of these did this method add anything to the x-ray evidence.

Clark (5), already quoted above, found that an increase of calcium in the blood stream by injection quickly reached the normal level; and that the ingestion of calcium did not affect the normal level in the blood. From these observations one is led to believe that the repetition of the present experiments with an attempt to increase the available calcium would be equally unsuccessful.

#### SUMMARY.

1. The average of twenty-six estimations of the normal inorganic phosphate in the whole blood of rabbits is 4.87 mg. of P per 100 cc.

2. The normal inorganic phosphate of rabbit's blood is practically—that is within biological limits—a constant.

3. An increase by four or five times in the concentration of inorganic phosphate in rabbit's blood returns to normal within 4 hours.

4. The intravenous injection of rabbits with 75 mg. of P per kilo of body weight in the form of  $\text{NaH}_2\text{PO}_4$  will cause tetany in a certain proportion of individuals.

5. On the basis of few experiments, the ingestion of cod liver oil causes no change in the level of inorganic blood phosphate in the rabbit and dog.

6. The intravenous injection in the rabbit of a single massive dose of phosphate has no demonstrable effect on the calcification of callus.

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# CAN "HOME GROWN RATIONS" SUPPLY PROTEINS OF ADEQUATE QUALITY AND QUANTITY FOR HIGH MILK PRODUCTION? III.\*

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Supplementary to previous work<sup>1</sup> we have continued our studies on the possibility of furnishing an adequate protein supply to high milk-producing cows from home grown sources. In the work done in 1919-20<sup>1</sup> with alfalfa hay, we maintained by the use of corn starch a like energy supply in the several rations when a change in the grain mixture was made. For example, when ground oats were substituted for ground corn meal the lowered net energy value of the ration was made good by the use of a definite amount of corn starch adjusted according to Armsby's<sup>2</sup> data in which he gives the net energy value of 100 pounds of corn meal as 89.16 therms and of 100 pounds of whole oats as 67.56 therms. When this was done, as our records showed, it was entirely possible to maintain nitrogen equilibrium and high milk production with these liberal milking animals over a period of 16 weeks. The possible effect of a lowered energy intake through the substitution of ground whole oats for ground corn meal in the ration with maintenance of a constant protein level but with no starch additions was now studied.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

<sup>1</sup> Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1919, xxxviii, 515; 1920, xlv, 189.

<sup>2</sup> Putney, F. S., and Armsby, H. P., *Pennsylvania State College Bull.* 143, 1916.

Such an experiment would touch practice more closely than our earlier experiments did, as the common farm procedure would be to use either a single grain such as whole oats or a mixture of grains, but without the addition of starch.

#### EXPERIMENTAL.

In these experiments we worked with the corn, barley, and oat grains, used singly and supplemented with corn silage and alfalfa hay. The alfalfa was grown in southern Wisconsin and was taken from the second cutting.

Since the protein content of barley was intermediate to that of the corn and oat grains used, we chose the barley ration as the standard to which the other rations must conform with respect to protein. For example, 50 pounds of the barley grain ration consisted of 10 pounds of grain, 10 pounds of alfalfa, and 30 pounds of corn silage. The corn grain mixture consisted of 10.6 pounds of corn meal, 10 pounds of alfalfa, and 30 pounds of silage and contained the same amount of total protein as the barley ration. The oat grain ration consisted of 9.1 pounds of whole oats, 10 pounds of alfalfa, and 30 pounds of silage and furnished the same amount of protein ( $N \times 6.25$ ) as the other rations. The 50 pounds of barley ration furnished 16.37 therms; the 50.6 pounds of corn ration furnished 17.24 therms; while the 49.1 pounds of oat grain ration furnished but 14.43 therms.

Much to our surprise these differences in the net energy values of the rations, particularly the oat ration as compared with the others, were sufficient to determine whether these high milk-producing cows would be in negative or positive nitrogen balance. The corn and barley rations were ample both in protein and net energy content for high milk production over the periods of observation; but the oat ration was not generous enough in its net energy content for these lactating animals—the effect being manifested by distinct negative nitrogen balances during the period of 4 weeks observation. Evidently protein was being destroyed as a source of energy during the feeding of this lower therm-containing ration.

Cow 3 weighed approximately 1,000 pounds and was producing daily 40 pounds of milk containing 3 per cent of fat. According to Armsby's standard such a cow would require for maintenance

and the production of this amount of milk a daily intake of 14.4 therms. Actually she was receiving 14.43 therms per day, but with that amount of energy, nitrogen equilibrium was not maintained; for while Armsby's standard requires 2.22 pounds daily of digestible *true* protein for a cow with the producing capacity of No. 3, she was receiving but 1.68 pounds of digestible *true* protein. The two other animals involved in this inquiry showed similar negative nitrogen balances on the whole oat ration but positive balances or equilibrium on the barley and corn rations, in which the protein content was the same as in the oat ration but the net energy supply 2 to 3 therms higher per day.

Animal 1 was a pure bred Holstein, No. 2 a grade Jersey, and No. 3 a grade Guernsey. They weighed respectively as follows: No. 1, initial weight 1,502 pounds, final weight 1,465 pounds; No. 2, initial weight 997 pounds, final weight 1,034 pounds; No. 3, initial weight 1,038 pounds, final weight 995 pounds. The methods of analysis and quantitative collection of excreta have been described in earlier publications. Each ration was fed for a period of 4 weeks with a preliminary feeding period of 5 days before quantitative collection of excreta was begun. There was no preliminary feeding period when the rations were changed. Each cow was allowed what she would completely consume of the mixed ration.

The ration used contained the following percentages of nitrogen:

	Nitrogen. per cent
Corn grain.....	1.57
Oat grain.....	1.82
Barley grain.....	1.66
Corn silage.....	0.36
Alfalfa hay.....	2.65

The data on nitrogen balances are presented in Tables I to III inclusive.

From the data presented in the tables it is clearly evident that there was not only a distinct negative nitrogen balance during the oat grain period of feeding, when the therm intake was reduced, but that there was a marked increased destruction of protein in this period as shown in the greatly increased urinary nitrogen output exhibited by each animal. No. 1 increased her urinary

nitrogen output 50 per cent above the urinary nitrogen output on the corn grain ration and increases of nearly similar magnitude were shown by Nos. 2 and 3.

These data are not to be interpreted as indicating an inferiority of the oat proteins as compared with those of the corn or barley grain since all available evidence<sup>1</sup> points towards an approximately equal supplementary efficiency for the cereal grain proteins.

TABLE I.

*Record of Nitrogen Balance, Milk Production, Etc., in Animal I.*

Date.	Nitrogen.					
	Intake.	Feccs.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	<i>gm.</i>	<i>gm</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>lbs.</i>
Dec. 14-20.....	1,712.9	647.7	570.9	585.8	-91.5	248
" 21-27.....	1,712.9	715.7	540.2	547.2	-90.2	236
" 28-Jan. 3.....	1,712.9	672.8	458.2	550.3	+31.6	241
Jan. 4-10.....	1,850.1	698.3	423.1	559.0	+169.7	244
Corn grain ration.						
Jan. 11-17.....	1,850.1	728.3	472.9	569.0	+79.9	252
" 18-24.....	1,850.1	788.9	436.8	569.6	+54.8	257
" 25-31.....	1,850.1	702.5	484.5	586.1	+77.0	258
Feb. 1-7.....	1,850.1	702.5	525.6	571.7	+50.3	252
Oat grain ration.						
Feb. 8-14.....	1,850.1	583.4	668.9	555.9	+41.9	244
" 15-21.....	1,850.1	616.5	707.0	556.9	-30.3	244
" 22-28.....	1,850.1	622.9	793.0	539.7	-105.5	236
Mar. 1-7.....	1,850.1	651.8	817.3	540.1	-159.1	232

Another important point coming from the collection of such data as here presented are differences in urinary nitrogen elimination which are often observed with approximately similar absorption from the intestine. Apparently in individuals different rates of deamination and destruction of important carbon nuclei are taking place which may be closely related to the often observed differences in the efficiency of a protein mixture with different animals. For example, in these trials No. 2 during the corn

ration period was absorbing approximately 1,000 gm. of nitrogen per week and eliminating 480 gm. in the urine, while Animal 3 during the same period was absorbing practically the same amount of nitrogen but eliminating only 410 gm. per week; presumably the energy requirement was amply covered in both individuals. It is in this direction; namely, the rates of intermediary nitrogen metabolism and the special tissues involved in producing these different rates, that we must look for an

TABLE II.  
*Record of Nitrogen Balance, Milk Production, Etc., in Animal II.*

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	gm.	gm.	gm.	gm.	gm.	lbs.
Dec. 14-20.....	1,575.7	549.6	409.2	491.3	+125.6	185
“ 21-27.....	1,575.7	588.1	524.3	444.5	+18.8	167
“ 28-Jan. 3.....	1,575.7	602.0	449.0	440.2	+84.5	188
Jan. 4-10.....	1,575.7	603.0	450.7	445.0	+77.0	194
Corn grain ration.						
Jan. 11-17.....	1,575.7	560.1	444.8	439.1	+131.7	180
“ 18-24.....	1,575.7	620.9	492.8	434.9	+27.1	160
“ 25-31.....	1,575.7	567.4	497.8	439.8	+70.7	185
Feb. 1-7.....	1,575.7	563.6	476.6	437.3	+98.2	180
Oat grain ration.						
Feb. 8-14.....	1,575.7	503.6	628.9	433.3	+9.9	177
“ 15-21.....	1,575.7	488.9	614.1	431.1	+41.6	173
“ 22-28.....	1,575.7	494.0	663.6	428.5	-10.4	174
Mar. 1-7.....	1,575.7	446.6	713.5	439.3	-23.7	175

explanation of differences in the efficiency of individuals in respect to protein utilization. While protein constitution is of primary importance in this respect, the additional factor of the individual rate of intermediary metabolism will come into play especially in the application to practice of a mathematical standard. This factor would assume special importance where the protein allowance was not liberal and was reduced to a standard formulated from data obtained with the “best” individuals.



TABLE III.  
*Record of Nitrogen Balance, Milk Production, Etc., in Animal III.*

Date.	Nitrogen.					
	Intake.	Feces.	Urine.	Milk.	Balance.	Milk per week.
Barley grain ration.						
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>lbs</i>
Dec. 14-20.....	1,712.9	664.2	415.5	638.7	-5.3	316
“ 21-27.....	1,712.9	661.2	387.8	612.8	+49.1	308
“ 28-Jan. 3.....	1,712.9	689.1	318.6	639.7	+65.5	341
Jan. 4-10.....	1,712.9	638.1	392.5	640.9	+41.4	334
Corn grain ration.						
Jan. 11-17.....	1,712.9	731.8	366.7	628.1	-13.7	317
“ 18-24.....	1,712.9	746.3	437.8	588.3	-59.5	321
“ 25-31.....	1,712.9	682.0	447.4	570.3	+13.2	319
Feb. 1-7.....	1,719.9	698.4	397.0	565.5	+52.0	308
Oat grain ration.						
Feb. 8-14.....	1,712.9	580.8	558.6	572.1	+1.4	302
“ 15-21.....	1,712.9	590.0	564.3	562.1	-3.5	291
“ 22-28.....	1,712.9	570.2	647.0	553.9	-58.2	278
Mar. 1-7.....	1,712.9	549.7	642.6	555.4	-34.8	279

TABLE IV.  
*Composition of Milk and Average Daily Flow in a Selected Week of Each Period.*

	Dec. 28	Jan. 25	Feb. 22
Animal I.			
Total solids, <i>per cent.</i> .....	12.98	12.03	11.85
Fat, <i>per cent.</i> .....	3.60	3.40	3.55
Nitrogen, <i>per cent.</i> .....	0.51	0.50	0.49
Milk daily, <i>lbs.</i> .....	35.00	35.00	34.00
Animal II.			
Total solids, <i>per cent.</i> .....	14.38	13.92	13.89
Fat, <i>per cent.</i> .....	4.80	4.70	4.70
Nitrogen, <i>per cent.</i> .....	0.52	0.53	0.51
Milk daily, <i>lbs.</i> .....	27.00	26.00	25.00
Animal III.			
Total solids, <i>per cent.</i> .....	11.75	10.79	10.84
Fat, <i>per cent.</i> .....	3.00	2.80	3.00
Nitrogen, <i>per cent.</i> .....	0.42	0.38	0.39
Milk daily, <i>lbs.</i> .....	48.00	45.00	40.00

In Table IV are recorded the composition of the milk and the average daily flow in a selected week of each period. The milk composition as well as flow was well maintained for the period of observation, but there can be no doubt but that a long continued feeding period on the oat ration, involving inadequate energy intake, would ultimately have affected milk secretion and milk composition.

#### SUMMARY.

1. Data are presented which show that it is entirely possible when feeding equal but limited amounts of protein to maintain nitrogen equilibrium and high milk production in dairy cows with a ration composed of either barley or corn supplemented with corn silage and alfalfa hay, but not with the whole oat grain so supplemented.

2. Previous records had indicated this possibility with all cereal grains, but only when the deficient net energy content of the oat grain ration was made good by the use of corn starch.

3. The oat grain ration contained 14.43 therms per 49.1 pounds; the corn ration contained 17.24 therms per 50.6 pounds; both rations contained exactly the same amount of protein; yet this difference in the energy supply of the two rations was sufficient to produce a positive nitrogen balance on the 17.24 therms but a negative nitrogen balance on the 14.43 therms.

4. In practice, a mixture of the corn grain or the barley grain with the oat grain, 50 per cent of each, would very probably make up this deficiency in net energy.



## STUDIES ON BLOOD SUGAR.

### THE TOTAL AMOUNT OF CIRCULATING SUGAR IN THE BLOOD IN DIABETES MELLITUS AND OTHER CONDITIONS.\*

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(Received for publication, July 18, 1921.)

In this paper observations are reported on the total amount of sugar in the circulating blood, and on its relative distribution between plasma and corpuscles in a series of normal persons, of diabetic patients, and of patients with other diseases.

Numerous experimenters have studied the distribution of sugar per unit volume of plasma and corpuscles. The subject was carefully reviewed by Gradwohl and Blaivas, in 1917, and more recently by Wishart. According to the statements of certain previous workers, the corpuscles sometimes contain little or no sugar; according to others, the sugar content of the corpuscles does not differ greatly from that of the plasma; another belief has been that the corpuscles take up sugar more slowly and retain it longer than does the plasma so that the corpuscular sugar is low in the early stages of hyperglycemia, but above that of the plasma in the declining stages. The preponderance of experience is that the sugar content per unit volume of corpuscles is usually a little below that of the plasma. According to Wishart the discrepancy in favor of the plasma generally becomes greater as the blood sugar rises.

The desirability of knowing the total amount of circulating sugar and its distribution between the total volume of plasma

\* This paper is Number 19 of a series of studies, on the physiology and pathology of the blood, from the Harvard Medical School and allied hospitals, a part of the expense of which has been defrayed from a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

and corpuscles was suggested by Epstein and Baehr in this country in 1914. These observers produced rapid changes in the blood volume of experimental animals. By diluting the blood the percentage concentration of sugar diminished, although the total amount of circulating sugar remained constant or was increased. They concluded, therefore, that in diabetes the total amount of circulating sugar must bear a more definite relation to glycosuria than the mere concentration of sugar for each unit volume of blood. Sansum and Woodyatt, in 1917, also determined the importance of the total blood sugar in establishing and regulating the rate of glycosuria and that the concentration of sugar in the plasma was of but little importance in this respect. No data have heretofore been published, however, which record the normal amount of circulating sugar in man, nor the variations from the normal that may occur in diabetes and other conditions. We obtained data on the subject by the following method.

The total volume of the circulating blood and the total corpuscular and plasma volume were determined by the vital red method of Keith, Rowntree, and Geraghty. One of us (Bock) has recently published a discussion of the advantages and disadvantages of this method. The sugar concentration of oxalated whole blood and oxalated unhemolyzed plasma for each 100 cc. was obtained at the same time by the method of Folin and Wu. Knowing the amount of sugar to 100 cc. of blood and plasma, and the total volume of circulating blood and plasma, the total amount of sugar in the whole blood and plasma was readily obtained. The differences between the total amount of sugar and that found in the plasma represented the total corpuscular sugar. The results of our observations are incorporated in Tables I to IV.

In Table I is recorded the total amount of sugar in the blood of seven normal persons; the amount varied between 7.54 and 2.50 gm. The plasma sugar varied between 4.85 and 1.64 gm. The corpuscular sugar varied between 3.85 and 0.69 gm. The average total amount of blood sugar for the group was 5.18 gm. The average plasma sugar was 3.29 gm. The average corpuscular sugar was 1.89 gm. The marked variations encountered in the individual cases probably depended, among other reasons, on wide differences in the area of body surface, and in weight, and on the fact that the estimations were made with

disregard for the time or nature of the previous meal. However, it seems logical to conclude from these data that by the methods employed the normal total amount of blood sugar does not exceed 7.50 gm., and that the normal plasma sugar is usually considerably greater than the corpuscular sugar, but does not exceed 4.85 gm.

In Table II are recorded similar observations in a group of nine diabetic patients. As was to be expected, in diabetics, as well as in normal persons, there were fluctuations in the amount of circulating sugar. The highest amount of sugar in the blood was 15 gm., the lowest 6.81 gm. The plasma, relatively

TABLE I.  
*Normal Persons.*

Case.	Sex.	Weight. <i>kg.</i>	Blood volume. <i>cc.</i>	Plasma volume. <i>cc.</i>	Corpuscular volume. <i>cc.</i>	Blood sugar mg. per 100 cc.		Total blood sugar. <i>gm.</i>	Total plasma sugar. <i>gm.</i>	Total corpuscu- lar sugar. <i>gm.</i>
						Blood.	Plasma.			
1	M.	82	6,218	3,731	2,487	120	130	7.54	4.85	2.69
2	M.	63	5,758	3,628	2,130	130	100	7.48	3.63	3.85
3	F.	55	5,750	3,970	1,780	100	90	5.75	3.55	2.20
4	F.	60	4,500	3,200	1,300	100	100	4.50	3.20	1.30
5	F.	60	4,460	3,120	1,330	90	100	3.81	3.12	0.69
6	F.	60	4,230	2,750	1,480	110	110	4.65	3.02	1.63
7	F.	60	3,570	2,340	1,230	70	70	2.50	1.64	0.86
Average.....								5.18	3.29	1.89

contained much more sugar than did the corpuscles. Thus the highest plasma sugar content was 10.78 gm., and the lowest 4.75 gm., while the highest corpuscular sugar content was 4.22 gm., and the lowest was 1.09 gm. The average total amount of blood sugar for the group was 8.95 gm. The average total plasma sugar was 6.72 gm. The average corpuscular sugar content was 2.23 gm.

These findings are evidence that the blood, especially the plasma in diabetes, is a vehicle for the transportation of sugar from the body cells which are unable to burn or store it, to the kidney which excretes it. The blood corpuscles, as a whole, are but

little concerned with such transportation of sugar and do not contain an increase in sugar proportional to that found in the plasma. Whether this depends largely, as Wishart has suggested, on the fact that glucose is more freely soluble in the plasma than in the corpuscular substance, or on other considerations, is uncertain. In any event, it seems logical to conclude with Wishart that analyses of plasma of the total amount of sugar and of its concentration for each 100 cc. are preferable to those of whole blood.

TABLE II.  
*Patients with Diabetes.*

Case.	Sex.	Weight. <i>kg.</i>	Blood volume. <i>cc.</i>	Plasma volume. <i>cc.</i>	Corpuscular volume. <i>cc.</i>	Blood sugar mg. per 100 cc.		Total blood sugar. <i>gm.</i>	Total plasma sugar. <i>gm.</i>	Total corpuscu- lar sugar. <i>gm.</i>
						Blood.	Plasma.			
1	F.	70.0	4,690	2,990	1,700	320	360	15.00	10.78	4.22
2	M.	47.0	4,030	2,500	1,530	170	190	6.86	4.75	2.11
3	M.	43.0	3,880	2,440	1,440	190	220	7.37	5.36	2.01
4	M.	41.5	3,784	2,365	1,419	180	220	6.81	5.20	1.61
5	M.	62.0	3,760	2,480	1,280	220	240	8.24	5.95	2.29
6	M.	54.0	3,590	2,370	1,220	200	250	7.18	5.92	1.26
7	M.	54.5	3,363	2,200	1,163	340	380	11.43	8.36	3.07
8	M.	50.0	3,340	2,140	1,200	240	260	8.02	5.56	2.46
9	F.	35.5	2,930	2,200	730	330	390	9.67	8.58	1.09
Average.....								8.95	6.72	2.23

The findings shown in Table II demonstrate, moreover, that blood sugar concentration expressed as mg. per 100 cc. of blood or plasma may give misleading information with regard to the total amount of circulating sugar, as Epstein and Baehr and Sansum and Woodyatt have suggested. For example, in Cases 1, 7, and 9, respectively, the sugar concentrations were 360, 380, and 390 mg. of sugar per 100 cc. of plasma. In Case 1 the plasma sugar concentration was the lowest of the three, with 10.8 gm. of sugar in the total plasma. In Case 7 the sugar in the total plasma was 8.36 gm., and in Case 9, in which the sugar concentration was the highest of the series, the sugar in the total plasma was only 8.6 gm.

The relation between the total amount of plasma sugar and the total amount of urine sugar was studied in a few cases. For this purpose the urine was collected for the 24 hour period in the middle of which the blood volume and sugar determinations were made. The sugar excretion was titrated by the Benedict method on the urine in cases which yielded a positive reaction to Benedict's qualitative test. The findings are recorded in Table III.

The threshold at which glucose appeared in the urine of patients with diabetes seemed to lie between 5.2 and 5.36 gm. of total plasma sugar. One diabetic patient whose plasma sugar concentration was 190 gm. per 100 cc. had a low plasma volume

TABLE III.

*The Relation between Total Plasma Sugar and Sugar Excretion.*

Case.	Diagnosis.	Plasma sugar mg per 100 cc.	Total plasma sugar.	Body weight. "W"	Urine sugar per liter. "C"	Urine sugar per 24 hours. "D"	Urine $\frac{D}{W} \sqrt{C}$
			gm.	kg.	gm.	gm.	
1	Normal.	130	4.85	82.0	0	0	
2	Diabetes.	190	4.75	47.0	0	0	
3	"	220	5.20	41.5	0	0	
4	"	220	5.36	43.0	Trace.	Trace.	
5	"	250	5.92	54.0	5.00	27.50	1.14
6	"	390	8.58	35.5	13.90	21.54	2.26
7	"	360	10.78	70.0	29.40	52.92	4.10

and thus a low total amount of sugar. This probably explains why the patient did not excrete sugar in the urine with so high a glycemia.

Three patients excreted titratable amounts of sugar. The total sugar excretion did not have any obvious connection with either the concentration of sugar in the plasma or with the total amount. The sugar excretion was therefore estimated according to the formula which Ambard has used in studying urea and chloride excretions and which Fitz and Van Slyke used as the basis of their acid excretion formula. Sugar excretion recorded in this manner was not proportional to the plasma sugar concentration. The total plasma sugar, however, appeared to be related to sugar excretion expressed in this form (Chart 1).



These observations are too few to be very conclusive. They suggest, however, that the total amount of plasma sugar offers a more rational basis of comparison with sugar excretion than does the plasma sugar concentration alone, and that it may be possible to work out a formula which will express mathematically the relationship between the sugar circulating in the blood and that excreted in the urine.

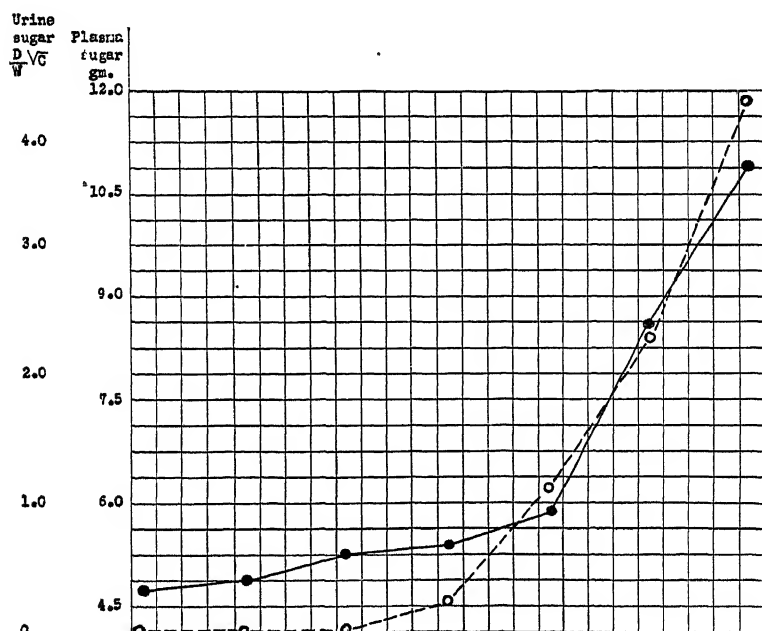


CHART 1. The relationship between total plasma sugar and the excretion of sugar in the urine.

The most striking feature of the miscellaneous cases tabulated in Table IV is the great difference in the estimation of total blood sugar. Thus a patient with polycythemia had 16.20 gm. of sugar in the blood, while a patient with nephritis had only 2.06 gm. of sugar in the blood. The average for this group of patients with miscellaneous diseases as a whole is lower than the average for normal persons (Table I), probably on account of the number of anemic patients who were studied.

It is of especial interest that the patient with polycythemia had more sugar circulating in the blood and more in the corpuscles than any of the diabetic patients, although the urine was normal. The total plasma sugar, on the other hand, was within normal limits. This suggests that sugar contained in the corpuscles is tightly bound to them in some manner and has little effect on the production of glycosuria.

In all three groups of cases the corpuscular sugar content was parallel with the corpuscular volume. Apparently, therefore,

TABLE IV.  
*Patients with Miscellaneous Diseases.*

Case.	Sex.	Weight. kg.	Diagnosis.	Blood volume. cc.	Plasma volume. cc.	Corpuscular vol- ume. cc.	Blood sugar mg. per 100 cc.		Total blood sugar. gm.	Total plasma sugar. gm.	Total corpuscular sugar. gm.
							Blood.	Plasma.			
1	M.	60.5	Polycythemia.	8,540	3,240	5,300	190	150	16.20	4.85	11.35
2		45.3	Leukemia.	5,060	3,460	1,590	90	80	4.55	2.77	1.78
3	F.	60.5	Secondary anemia.	4,470	3,400	1,070	110	110	4.92	3.76	1.16
4	M.	65.0	Nephritis.	4,390	3,170	1,270	110	80	4.83	2.54	2.29
5		50.0	Leukemia.	4,030	3,140	890	100	100	4.03	3.14	0.89
6	F.	43.5	Pernicious anemia.	3,070	2,730	340	120	120	3.68	3.28	0.40
7	F.	43.5	" "	3,050	2,600	460	120	120	3.66	2.60	1.06
8	F.	54.5	" "	2,740	2,330	410	120	120	3.29	2.80	0.49
9	M.	54.5	Nephritis.	2,680	2,140	530	80	90	2.14	1.92	0.22
10	M.	45.0	"	2,580	2,200	380	80	80	2.06	1.76	0.30
11	F.		Pernicious anemia.	2,460	2,180	280	100	90	2.46	1.96	0.50
Average.....									4.71	2.85	1.86

all blood corpuscles contain a certain amount of sugar which is fixed within rough limits. If the number of corpuscles is greatly increased, the total corpuscular sugar is also increased; the corpuscular content of sugar is low when the number of corpuscles is diminished; when sugar is added to the circulating blood it is found largely in the plasma and to a much less extent in the corpuscles. Glycosuria does not occur unless the total plasma sugar is above a certain limit, regardless of what the sugar content of the corpuscles or whole blood may be.

## SUMMARY.

The total amount of sugar in the blood of seven normal persons varied but did not exceed 7.5 gm. The plasma sugar was almost always considerably greater than the corpuscular sugar, but it did not exceed 4.85 gm. The total amount of sugar in the blood of nine diabetic patients also varied considerably. The highest blood sugar content estimated was 15 gm., and the highest plasma sugar was 10.78 gm.

The plasma of the diabetic bloods, relatively, contained much more sugar than did the corpuscles. This suggests that the plasma in diabetes is a vehicle for the transportation of sugar from the body cells, which are unable to burn or store it, to the kidney which excretes it, and that the blood corpuscles are but little concerned with such transportation of sugar, a statement which is supported by the fact that the sugar content of the individual corpuscle tends to be fixed within rough limits. If the number of corpuscles is increased, as in polycythemia, the total corpuscular sugar is increased. If the number of corpuscles is much diminished, as in anemia, the amount of corpuscular sugar is diminished. Glycosuria does not occur unless the plasma sugar exceeds a certain threshold.

Blood sugar concentration expressed as mg. per 100 cc. of blood or plasma may give misleading information with regard to the total amount of circulating sugar. The threshold at which glucose appeared in the urine of the diabetic patients of this series seemed to lie between 5.20 and 5.36 gm. of total plasma sugar. The total plasma sugar offered a more rational basis of comparison with sugar excretion than did the plasma sugar concentration alone.

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## EFFECT OF HEATING THE ANTISCORBUTIC VITAMINE IN THE PRESENCE OF INVERTASE.

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A number of papers which have appeared recently have suggested that the presence of enzymes in food may influence the stability of the antiscorbutic enzyme. Givens and McClugage (1) state:

"The influence of heat upon the antiscorbutic vitamine appears to be related not only to the degree of temperature but to the duration of the treatment, the reaction, the enzymes present, and the manner of heating."

They base their conclusion as to this enzyme action partly upon experiments showing that potatoes baked for a short time at high temperature and then dried at 35-40°C. retain a greater amount of the antiscorbutic vitamine than those dried at 35-40°C. without previous heating.

In this same article, Givens and McClugage refer especially to oxidases present in the potato. Ellis, Steenbock, and Hart (2) have shown that drying cabbage in an atmosphere of CO<sub>2</sub> for 35 hours at 65°C. does not prevent the destruction of the antiscorbutic vitamine. Oxidizing agents such as hydrogen peroxide and potassium permanganate cause its destruction.

Anderson, Dutcher, Eck'es, and Wilbur (3) state that oxidation is a more important factor than heat in the destruction of the antiscorbutic vitamine. Bubbling air through cow's milk at 145°F. for 30 minutes causes some destruction, but the destruction is more marked when oxygen or hydrogen peroxide is used.

These experiments, together with the fact, as shown by Harden and Robinson (4) and Givens and Macy (5), that dehydrated orange juice retains its antiscorbutic value after 2 years, whereas untreated orange juice deteriorates appreciably within 3 months (6) indicate strongly that enzyme action may be an important factor in the gradual destruction of the antiscorbutic vitamine.

## EXPERIMENTAL.

In the present study the authors have heated orange juice, both in the presence and absence of an enzyme. The temperatures chosen were 38°, 55°, and 76°C. If enzyme activity causes destruction of the vitamine, presumably disappearance of the latter would be most rapid at 55°C., the temperature at which the activity of the enzyme is greatest.

The enzyme selected was invertase, since it is present in the natural orange juice. The experiments conducted show conclusively that this enzyme does not decrease the value of the antiscorbutic vitamine. This does not preclude the possibility that other enzymes may have such an effect, and it is hoped that experiments along this line may be continued with an oxidizing enzyme.

The antiscorbutic vitamine was separated from orange juice<sup>1</sup> by the method of Hess and Unger (6) with 96 per cent alcohol which precipitated the enzyme and thus separated it from the vitamine. The extract was tested on a solution of sucrose and gave a negative test for invertase.

The invertase was prepared by a method described by Hudson and Paine (7). It was dried on filter paper and 25 mg. were added to each 100 cc. of orange extract.

4 cc. of orange extract, prepared fresh about every 10 days, were fed to each guinea pig daily for the first 46 days of the experiment. As none of the guinea pigs receiving the extract showed any symptoms of scurvy at that time, the amount fed was reduced to 3 cc. for the following 18 days. As there were still no indications of scurvy in any of the animals, and as the experiment had to be terminated within another month, the amount fed daily was reduced to 1.5 cc. per animal.

Table I gives the amount fed for the various periods, and the time each portion was heated.

The basal diet used, consisted of equal parts by weight of alfalfa meal and wheat flour, with powdered milk which had previously been heated to 95°C. for 1 hour. These three were mixed thoroughly together and made into a soft paste with water.

<sup>1</sup> The oranges in these experiments were generously supplied by the Research Laboratory of the California Fruit Growers Exchange, Corona, California.

The allowance of milk was about 3 cc. per day per animal. Cracked oats were kept constantly before the animals.

Table II gives the diets fed the various guinea pigs.

The animals were weighed every morning before feeding. In Table III are recorded the weights for every fifth day.

TABLE I.  
*Treatment of Orange Extract.*

Time heated.	Amount fed.	Period fed.
<i>hrs.</i>	<i>cc.</i>	<i>days</i>
4	4	11
2	4	21
4	4	15
4	3	18
4	1.5	25

TABLE II.  
*Diets.*

No. of animal.	Basal diet.	Invertase.	Orange extract.	Temperature heated.
				$^{\circ}\text{C}.$
29	+	—	—	
25	+	+	—	
27	+	—	+	
33 and 34	+	—	+	38
31 and 37	+	+	+	38
28 and 35	+	—	+	55
32 and 36	+	+	+	55
30 and 38	+	—	+	76
26 and 39	+	+	+	76

#### SUMMARY.

Invertase does not contribute to the destruction of the anti-scorbutic vitamine when heated with the vitamine for 4 hours at  $76^{\circ}$ ,  $55^{\circ}$ , or  $38^{\circ}\text{C}.$

Heating for 4 hours at a temperature of  $76^{\circ}\text{C}.$  either in the presence of invertase or in its absence, causes a more rapid destruction of the vitamine than heating at  $55^{\circ}\text{C}.$  Heating for 4 hours at  $38^{\circ}\text{C}.$ , does not cause an appreciably greater loss of antiscorbutic value than keeping at room temperature.





In all cases except Guinea pig 32 which was fed on orange extract plus invertase heated at 55°C., the animals receiving orange extract heated in the presence of the enzyme were in a less advanced stage of scurvy at the close of the experiment than those receiving orange extract similarly heated without the enzyme. The authors have at present no suggestion as to the significance of this fact.

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# THE EFFECT OF CERTAIN STIMULATING SUBSTANCES ON THE INVERTASE ACTIVITY OF YEAST.

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The remarkable effect on yeast growth produced by the addition of an alcohol or water extract of yeast to a synthetic medium has been noted by several investigators. Wildiers<sup>1</sup> in 1901 first observed this fact. More recently others have confirmed Wildier's observations.

Euler<sup>2</sup> in studying invertase formation in yeast, found that the "Generationsdauer"—that is, the time required for yeast cells to double in number—was only one-half as long when a yeast extract was added to the nutrient medium as when other sources of nitrogen were used. Equivalent amounts of asparagine, glycocoll, alanine, cystine, and tyrosine were added singly or in combination, and all proved less favorable for yeast growth than the yeast extract.

Williams<sup>3</sup> presented evidence that the constituent of yeast which stimulates reproduction of yeast cells is identical with the antineuritic vitamine, and that the rate of reproduction of yeast cells might be used as a quantitative method for determining this substance. Almost simultaneously, Abderhalden and Koehler,<sup>4</sup> using the same method—microscopic observation of the growth of single yeast cells in hanging drops—found that the addition of dilute extracts of yeast markedly accelerated the rate of reproduction. Similar results were obtained with two other organisms, *Colpoda cucullus* and the alga, *Ulothrix*. These authors also

<sup>1</sup> Wildiers, E., *La Cellule*, 1901, xviii, 313.

<sup>2</sup> Euler, H., *Biochem. Z.*, 1918, lxxxv, 406.

<sup>3</sup> Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

<sup>4</sup> Abderhalden, E., and Koehler, A., *Arch. ges. Physiol.*, 1919, clxxvi, 209.

suggest that the active substance is the antineuritic vitamine and that yeast or some other simple organism might be used to determine its presence.

It is reasonable to suppose that a substance so potent in its effect on higher organisms as the antineuritic vitamine is known to be must also play a significant rôle in the life processes of the yeast cell itself. The effect of the presence or absence of such a necessary substance on a simple cell like yeast can be more easily studied than in the more complex forms of life, and a knowledge of its function in the yeast cell undoubtedly is of fundamental importance for an understanding of its mode of action in higher organisms.

Abderhalden and Schaumann<sup>5</sup> have suggested the significance of this phase of yeast nutrition. They prepared an active extract by treating yeast with 10 per cent  $I_2 \cdot C_4$  for 24 hours. After filtering and removing the  $I_2 \cdot C_4$  with barium the solution was evaporated to dryness and the residue extracted with absolute alcohol five times. The combined alcohol extracts were evaporated under reduced pressure and the residue again extracted with absolute alcohol. This process was repeated until the residue was entirely soluble in alcohol. The alcohol solution was finally evaporated and the residue taken up in water. Such a yeast extract contained 8.95 per cent dry substance, 0.87 per cent ash, 0.19 per cent nitrogen, and 0.093 per cent phosphorus. Additions of 5 to 10 cc. of this extract to 250 cc. of a sugar solution in which living yeast was suspended accelerated greatly the fermentation of glucose, fructose, galactose, sucrose, and maltose. This action seemed to be specific for some unknown substance in the yeast extract in that amino-acids and phosphorus compounds, added to the fermentation mixtures, were entirely inactive. The  $H^+$  concentration was kept constant so as to eliminate that as a factor in the increased rate of fermentation.

A more highly purified preparation which they call "Eutonin" was precipitated from the alcohol extract by acetone. This was entirely phosphorus-free, but was still effective in augmenting fermentation by living yeast, although it was not so active as the original extract. These results indicate that in the "crude" extract more than one substance was active.

<sup>5</sup> Abderhalden, E., and Schaumann, H., *Fermentforschung*, 1919, ii, 120.

These questions arise: To what is the action of the yeast extract due? Does its effect lie solely in accelerating cell reproduction? Does it stimulate the formation of one or more of the enzymes? Or, finally, does it act directly on the enzymes as an activator or as a coenzyme?

Abderhalden and Schaumann state that the addition of yeast extract had some effect in accelerating fermentation when dried yeast or a maceration juice was substituted for the living yeast. On the basis of their results they conclude that there is a substance in the yeast extract which acts as a coenzyme or activator of an enzyme. However, their protocols show that in the experiments in which dried yeast or maceration juice was used, only slight increases in the rate of fermentation followed the addition of the yeast extract. As the determination of loss in weight was the method used for measuring the rate of fermentation the small increases in fermentation which were found in these experiments might reasonably come within the limits of experimental error. Also, there was opportunity for bacterial growth during the long period in which these mixtures were allowed to ferment without the addition of toluene.

When living yeast was used there was undoubtedly a remarkable increase in the rate of fermentation with the addition of yeast extract to the fermentation mixture. This increase occurred, too, after the tenth hour, during the period of most rapid growth of yeast.<sup>6</sup> It appears then that the chief effect of the active substance in yeast extract was on the living yeast, either in promoting growth or in stimulating the production of one or more of the enzymes.

Euler<sup>7</sup> and other workers have reported the variations in invertase activity of yeast grown in different media. Not only the substrate, sucrose, and the reaction products, glucose and fructose, but other hexoses as well, especially mannose, increase the formation of invertase.

Euler<sup>2</sup> also investigated the effect of nitrogen nutrition on invertase formation in yeast. He used  $(\text{NH}_4)_2\text{SO}_4$ , asparagine, glycocoll, alanine, tyrosine, and cystine as sources of nitrogen,

<sup>6</sup> Euler, H., and Lindner, P., *Chemie der Hefe und der alkoholischen Gärung*, Leipsic, 1915, 254.

<sup>7</sup> Euler, H., and Cramér, H., *Biochem. Z.*, 1913-14, lviii, 467.

the amino-acids having been added to the medium both singly and in combination. All these nitrogen compounds were about equal in their effect on growth and on the invertase activity of the yeast. However, when an equivalent amount of a water extract from yeast was used as a source of nitrogen, there was a decided increase both in the rate of growth and in the formation of invertase. Euler ascribes this effect to a nitrogenous substance in the yeast extract which is in the most available form for yeast nutrition. But here again it is obvious that some other stimulating substance present in the yeast extract may be responsible for these results.

The experiments reported in this paper were undertaken prior to the writer's knowledge of the Abderhalden work, and were devised to obtain further information concerning the effect of yeast extract on the enzymes of yeast. To begin with, invertase was chosen as the enzyme, the rate of formation of which was to be followed, because its activity is so easily measured and because it is so important in yeast action.

As the work progressed the question soon came up as to whether the substance which promotes growth of yeast also accelerates invertase formation. Yeast was grown with and without addition of solutions known to contain the growth stimulant. Various methods were used to separate this substance from other constituents and these partially purified preparations were also added to the standard medium. The yeast grown in these different media was filtered off and the invertase activity thereof determined.

#### EXPERIMENTAL.

##### *Method for Determining Invertase in Small Amounts of Yeast.*

For this work it was first necessary to devise a method for extracting invertase quantitatively from small amounts of yeast. Euler's method was as follows.<sup>2</sup> The filtered yeast was drained a few minutes on a porous plate, 0.25 gm. portions were weighed out and suspended in 10 cc. of 1 per cent  $\text{NaH}_2\text{PO}_4$  solution, and after standing 10 minutes this suspension was added to 20 cc. of 20 per cent cane-sugar solution. At stated times samples were removed to determine the rotation. The inversion was checked

and multirotation accelerated by adding 10 cc. of 5 per cent NaOH solution. Lövgren<sup>8</sup> used practically the same method.

A serious objection to this method is that the yeast cells are not killed and when inversion was allowed to continue several hours some growth and fermentation might occur. To obviate this, various methods of killing the yeast cell and extracting the enzyme were tried. Small amounts of yeast, 0.1 to 0.3 gm., were dried at 37° for 2 to 4 hours, then shaken with water to which a few drops of toluene were added. Other samples were ground in a mortar with fine sand or other abrasive material before treating with water. None of these methods gave uniform results.

Finally the method of Willstätter, Oppenheimer, and Steibelt<sup>9</sup> for the quantitative determination of maltase was adopted for our purpose. Fresh yeast was shaken with water and toluene. Willstätter and coworkers obtained almost complete extraction of maltase in 24 hours. There was a slight increase by 48 hours treatment. Because maltase is rapidly destroyed by acid, it was necessary to keep the extract neutral by frequent additions of  $\text{NH}_4\text{OH}$ . With invertase this was unnecessary since this enzyme is more resistant to acid and is also most active in faint acidity.

Invertase is more readily extracted by this method than is maltase. Fresh samples of Fleischmann's yeast were weighed out, suspended in 25 cc. of water, 0.5 cc. of acid-free toluene was added, and the mixtures were shaken moderately at 30° for 4, 8, 12, and 24 hours respectively. At the end of the stated time each mixture was transferred to a 100 cc. volumetric flask. 50 cc. of 20 per cent sucrose solution, 0.4 cc. of 0.1 N HCl, and water to fill to the mark, were added. This was put into a 500 cc. Erlenmeyer flask and mixed well. 50 cc. were then removed, and 2 drops of  $\text{NH}_4\text{OH}$  (sp. gr. 0.90) added to check invertase action and to hasten multirotation. Before filtering it was necessary to add a small amount of talcum in order to obtain a perfectly clear filtrate. The rotation of the clear filtrate was then read in a 2 dm. tube at 20–25°C.

<sup>8</sup> Lövgren, S., *Fermentforschung*, 1919–20, iii, 221.

<sup>9</sup> Willstätter, R., Oppenheimer, T., and Steibelt, W., *Z. physiol. Chem.*, 1920, cx, 232.



The remaining 50 cc. were moderately shaken at 30°C. for 7 hours, after which the solution was made alkaline, filtered, and the rotation noted in the same way.

The results are given in Table I. It is evident that most of the activity was obtained by a preliminary treatment of 4 hours. There was a slight increase up to 12 hours, but a marked diminution in 24 hours.

TABLE I.

Weight of Fleischmann's yeast taken.	Time of shaking.	Change in $\alpha$ in 7 hrs.
<i>gm.</i>	<i>hrs.</i>	
0.2	4	11.88
0.2	8	11.84
0.2	12	12.38
0.2	24	8.36
0.3	4	14.90
0.3	8	14.98
0.3	12	15.24
0.3	24	12.71

As a final check on the method the invertase activity was determined on samples of yeast which were weighed out by another person. These results are found in Table II.

TABLE II.

Weight of Fleischmann's yeast taken.	Time of shaking.	Change in $\alpha$ in 7 hrs.
<i>gm.</i>	<i>hrs.</i>	
0.15	12	8.7
0.2	12	12.36
0.3	12	15.26

For the subsequent determinations the yeast was shaken with water and toluene at 30°C. for 12 hours—from 8.00 p.m. to 8.00 a.m.—then added to the sugar solution, and the change in rotation taken at the end of 7 hours. The toluene prevented any bacterial action.

It was found that the invertase extract was optically inactive. For convenience in taking the initial reading a blank was made up of 50 cc. of a 20 per cent sucrose solution plus 0.2 cc. 0.1 N HCl and water to make 100 cc. The rotation of this solution

was immediately taken for the initial reading. In the earliest studies the solutions were brought to a temperature of 20° before the rotations were read. The differences obtained in the readings with different samples of yeast were so great, however, that the slight errors due to a difference of 2 or 3° temperature were insignificant. Subsequent determinations were made at room temperature, which varied from 22–23°.

*Changes in Invertase Activity of Yeast Grown with the Addition of an Alcohol Extract of Yeast.*—An alcohol extract of yeast was prepared as follows: 3 pounds of starch-free Fleischmann's yeast were broken into fine pieces and dried in a current of warm air for 48 hours. The dry yeast was then covered with ether and heated on a water bath under a reflex condenser. This was repeated three times. Five extractions with 70 per cent alcohol were made in the same way. The alcohol extraction was continued over 2 days. The combined alcohol extracts were equal to 1,850 cc.

200 cc. of this alcohol extract were evaporated on the steam bath and the residue extracted with warm water four times. There was considerable lipin material present, so that in order to obtain a clear filtrate the water extract was run through a Berkefeld filter. The filtrate was made up to 200 cc. in water and labeled Alcohol Extract I.

Portions of this preparation were added to the medium in which yeast was grown to determine whether such an addition actually does increase the invertase activity of the yeast.

The medium used for the growth of the yeast was that adopted by Williams. It contained per liter

20.0	gm. of sucrose.
1.5	" " asparagine.
3.0	" " $(\text{NH}_4)_2\text{SO}_4$
2.0	" " $\text{KH}_2\text{PO}_4$
0.25	" " $\text{CaCl}_2$ .
0.25	" " $\text{MgSO}_4$ .

500 cc. portions of this medium were introduced into each of four 2 liter conical flasks. This exposed a large surface and insured sufficient oxygen for vigorous growth. To two of the flasks were added 2.5 cc. of the alcohol extract plus 2.5 cc. of water, and to the other flasks 5 cc. of the same alcohol extract. These mixtures were sterilized at 10 pounds pressure for 15 minutes.

A yeast suspension was prepared by shaking 0.2 gm. of Fleischmann's yeast in a liter of distilled water. 5 cc. of this suspension were then introduced into each of the flasks with a sterile pipette. The flasks were then incubated at 30° for 24 hours. It was evident that growth was much greater in the flasks to which 5 cc. of alcohol extract had been added.

The yeast was then filtered by suction, onto alundum crucibles, washed twice with distilled water, and sucked dry. To facilitate the filtering, the contents of the flasks were first centrifuged and

TABLE III.

*Changes in Invertase Activity of Yeast Grown with the Addition of Alcohol Extract I. 0.2 Gm. (Moist Weight) Used in Each Invertase Test.*

Yeast grown with addition of extract.	Change in $\alpha$ in 7 hrs.
cc.	
2.5	4.31
	4.54
5	8.91
5	9.25
	9.73
2.5	5.38
	5.50
2.5	4.51
	4.36
5	7.08
	6.93
5	7.38
	6.79

most of the supernatant liquid was poured off. The yeast was then washed into the crucibles.

0.2 gmi. portions of each sample of yeast were weighed out in duplicate, washed into Erlenmeyer flasks with 25 cc. of water, 0.5 cc. of toluene was added, and the mixtures were shaken 12 hours. The invertase preparation was then combined with the 20 per cent sucrose solution, 0.4 cc. of 0.1 N HCl, and water to make 100 cc. The initial rotation and the rotation at the end of 7 hours were taken as described.

These results are given in Table III. There is no doubt that the alcohol extract not only increases the rate of growth, but

also the invertase activity per unit weight of yeast. In this case when twice the amount of alcohol extract was added to the medium the invertase activity was doubled. This experiment was repeated a second time with similar results. Dry weights of these yeast samples were not obtained. However, the differences in moisture content could not account for the great differences in invertase activity of the different preparations. In subsequent experiments dry weights were determined and were found to be quite uniform in a single experiment.

The next step was to determine whether the substance which stimulates cell reproduction is identical with the substance which stimulates invertase formation. McCollum and Simmonds<sup>10</sup> have shown that the water-soluble vitamine is soluble in benzene after it has once been dissolved in alcohol. This suggested itself as a method of obtaining evidence as to whether the growth-promoting substance was also responsible for the increase in invertase formation.

*Invertase Activity of Yeast Grown with the Addition of Benzene-Soluble Material.*

200 cc. of the alcohol extract of yeast, equivalent to 2.74 gm. of total solid, were evaporated at 37° on 15 gm. of starch. This material was extracted with anhydrous ether for 6 hours to remove lipins. After this it was extracted with three portions of redistilled benzene (Kahlbaum's) for periods of 6, 7, and 8 hours. These combined benzene extracts were evaporated on a water bath and the residue was extracted with 200 cc. of water. This was called Benzene Extract I. It contained only 0.019 per cent of total solids and 0.0014 per cent of nitrogen. Yet when compared with Alcohol Extract I it was shown to be fully one-half as active in promoting growth (see Table IV).

5 and 10 cc. portions of the aqueous solution of this benzene extract were added to 500 cc. of the standard medium and 5 cc. of a yeast suspension introduced. The 24 hour growth of yeast was tested for its invertase activity as described above. The results are found in Table V.

<sup>10</sup> McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

TABLE IV.  
*Comparison of Growth-Promoting Activity of Various Preparations.*

Extract used.	Growth of yeast cells per 1 cc. added to 30 cc. of medium.
Alcohol Extract I.....	469
“ “ I.....	424
	446
Benzene Extract.....	198
“ “ .....	230
	214
Alcohol Extract of benzene in soluble material.....	212
“ “ “ “ “ “ .....	190
	201
Fullers' Earth Filtrate.....	34
“ “ “ .....	28
	31
“ “ Extract.....	91
“ “ “ .....	107
	99
Alcohol Extract II.....	418
“ “ II.....	550
	484
Phosphotungstic Acid Filtrate.....	80
“ “ “ “ .....	94
	87
“ “ “ I.....	30
	30
“ “ “ II.....	11
“ “ “ II.....	12
	11
Wheat Germ Extract.....	482
“ “ “ .....	492
	487

TABLE V.  
*Addition of Benzene Extract of Alcohol-Soluble Material from Yeast.*

Yeast grown with addition of benzene extract.	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
cc.		per cent
5	2.425	15.8
	2.405	
5	2.525	14.5
10	2.34	15.6
10	2.405	15.1
20	2.63	23.2
20	2.81	
20	2.55	

Here, contrary to the expected results, the addition of increasing amounts of benzene extract had no effect on the invertase activity, although the growth was comparable to the amount of extract added.

*Effect of Adding Material Soluble in Alcohol after Benzene Extraction.*—The residue after benzene extraction was reextracted with alcohol for 7 hours, and the residue from this alcohol extract dissolved in 200 cc. of water. This in turn was added to the medium and yeast grown in it. As seen by the data given in Table VI, the substance which increases invertase concentration in the yeast has been recovered in this solution. By the benzene extraction there has been a separation of the growth stimu-

TABLE VI.

*Addition of Material Soluble in Alcohol Following a Thorough Extraction by Benzene.*

Yeast grown with addition of extract.	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
cc.		<i>per cent</i>
5	7.40	26.7
	7.37	
5	10.83	26.8
	11.59	
20	16.93	25.1
	16.85	
20	16.89	25.5
	16.98	

lant from the substance responsible for the formation of the enzyme.

To confirm these results two other methods of separating the two substances were tried—precipitation with phosphotungstic acid and shaking with fullers' earth.

*Separation by Fullers' Earth.*—300 cc. of the alcohol extract of yeast were evaporated on the water bath and the residue was extracted with 200 cc. of water. After filtering, 100 cc. of this solution were shaken for 1 hour with 20 gm. of fullers' earth (Eimer and Amend). The earth was filtered off and washed once with water. The filtrate was made up to 150 cc. and called Fullers' Earth Filtrate.

The fullers' earth was shaken with 100 cc. of saturated  $\text{Ba}(\text{OH})_2$  for 10 minutes. The barium was removed from the combined filtrates with  $\text{H}_2\text{SO}_4$ . After concentrating on the water bath  $\text{BaSO}_4$  was filtered off, washed well, and the filtrate made up to 150 cc. This was called Fullers' Earth Extract.

A comparison of the activity of the filtrate and extract in promoting growth (by the counting method) shows the latter to be much more active (see Table IV). But here again the rate of growth and the rate of invertase formation do not run parallel.

Yeast grown with addition of 15 cc. of the Earth Extract was found to have almost identically the same invertase activity as yeast grown in standard medium with addition of only 15 cc. of distilled water. The rate of growth, however, was very different in the two cases. On the blanks, to which water was added, the growth from six flasks was required to yield 0.37 gm. of moist yeast (0.1354 gm. of dry weight). The addition of 15 cc. of Earth Extract produced a growth of 0.67 gm. of moist yeast (0.1687 gm. of dry weight) in two flasks.

On the other hand the addition of 15 cc. of Earth Filtrate to 500 cc. of medium yielded 0.965 gm. of moist yeast (0.2576 gm. of dry weight) in three flasks as compared with 1.8 gm. of moist yeast (0.5292 gm. of dry weight), the growth in two flasks with the addition of 15 cc. of the original alcohol extract. Yet the invertase activity in these two samples of yeast is of a similar order, being even a little greater in the yeast grown with the addition of the Earth Filtrate (see Table VII).

*Separation by Phosphotungstic Acid Precipitation.*—Williams was able to precipitate with phosphotungstic acid the substance which promotes yeast growth. An attempt was made to repeat this experiment as a third method of showing that the growth-promoting substance is not identical with the one which affects invertase formation.

150 cc. of a water solution of Alcohol Extract II (prepared as described for Alcohol Extract I) was evaporated to 20 cc. This concentrated solution was acidified to 5 per cent with  $\text{H}_2\text{SO}_4$  and 50 cc. of a 20 per cent solution of phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$  were added. This was an excess of about 20 cc. of phosphotungstic acid. It was allowed to stand in the ice box over night. The precipitate was then filtered off by suction and

washed twice with 10 per cent phosphotungstic acid in 5 per cent  $\text{H}_2\text{SO}_4$ .

The filtrate was freed from phosphotungstic acid by shaking in a Squibb separatory funnel with a mixture of equal parts of amyl alcohol and ether.  $\text{H}_2\text{SO}_4$  and the last traces of phosphotungstic acid were removed from the filtrate by adding a slight excess of  $\text{Ba}(\text{OH})_2$  and filtering. The precipitate was well washed and the filtrate made slightly acid with  $\text{H}_2\text{SO}_4$ , after which it was concentrated on the water bath to 150 cc., again neutralized and filtered. This was called Phosphotungstic Acid Filtrate.

The precipitates were suspended in 75 cc. of 5 per cent  $\text{H}_2\text{SO}_4$  and decomposed by shaking with the amyl alcohol-ether mixture.

TABLE VII.

*Separation of Growth Stimulant from Substance which Stimulates Production of Invertase by Means of Fullers' Earth.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.	
		per cent	
15 cc. Original Alcohol Extract I.....	6.39	27.5	29.4
	6.55	31.3	
15 " Earth Filtrate.....	7.63	26.9	26.8
	8.99	26.6	
15 " " Extract.....	3.23	25.5	25.1
	4.35	24.7	
15 " water.....	3.05	36.6	

Only part of the precipitates could be decomposed in this manner. The remaining precipitate was filtered off and decomposed in the usual manner by treating with saturated  $\text{Ba}(\text{OH})_2$  solution. The barium was then precipitated with  $\text{H}_2\text{SO}_4$ . The filtrate from the  $\text{BaSO}_4$  was diluted to 150 cc. and called Phosphotungstic Acid Precipitate II.

The acid solution of precipitates decomposed by amyl alcohol-ether treatment was neutralized with  $\text{Ba}(\text{OH})_2$  solution. This filtrate was made up to 150 cc. and called Phosphotungstic Acid Precipitate I.

Williams<sup>3</sup> found that he recovered from the phosphotungstic precipitate even greater activity in growth promotion than was



present in the original solution. He ascribes this to the effect of acid hydrolysis. The writer was unable to obtain such results. In two attempts to separate the growth stimulant by phosphotungstic precipitation a great loss in activity was observed (see Table IV). Also, the addition of the phosphotungstic acid filtrate produced greater growth than either one of the decomposed precipitates or even than the combined precipitates. As yet it is not known just where this loss of activity occurred.

There was no loss, however, in the substance which accelerated invertase formation. That remained almost entirely in the filtrate, as may be seen by referring to Table VIII.

*A gummy precipitate which separated from the original hot alcohol extract on cooling contained the substance active in invertase formation in high concentration.* A water solution made of a small amount of this precipitate was compared with Alcohol Extract II for activity both in promoting growth and in stimulating invertase formation. The data are given in Table IX. The solution of the precipitate although only one-tenth as active in increasing growth was equal in its effect on invertase formation.

This is further evidence that the active substance in promoting reproduction of yeast cells, whether it is the water-soluble vitamine or a stimulant specific for yeast, is not the constituent which affects the invertase concentration of the yeast.

*Alcohol Extract of Wheat Germ.*—Having found in yeast extract a substance so potent in accelerating invertase formation in yeast during growth, it seemed of interest to investigate extracts of other materials known to be rich in the growth stimulant. Thus far the only preparation we have used is an alcohol extract of wheat germ. 50 gm. of wheat germ were dried in a vacuum desiccator over  $\text{CaCl}_2$  for 3 days. This was extracted with ether for 6 hours, followed by extraction with alcohol for three periods of 6, 6, and 8 hours, using fresh portions of alcohol each time.

100 cc. of a water solution were made up equivalent to 15 gm. of the wheat germ. This was very active in promoting growth (see Table IV). Yet, addition of this preparation had apparently no effect on the invertase formation in the yeast. As seen by data given in Table X, increasing the amount of wheat germ extract by four times had no effect in stimulating greater invertase production.

TABLE VIII.

*Separation of Growth Stimulant from Substance Which Stimulates the Production of Invertase by Phosphotungstic Acid Precipitation.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.
		<i>per cent</i>
10 cc. Original Alcohol Extract II.....	5.44 5.51	26.9
10 " Filtrate from Phosphotungstic Precipitate .....	5.31 5.41	26.5
10 " Precipitate I.....	1.83 1.97	28.4
10 " " II.....	1.42 1.34	27.6
10 " water.....	0.57 0.49	26.8

TABLE IX.

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in the moist yeast.	Growth of yeast cells per 1 cc.
		<i>per cent</i>	
10 cc. Alcohol Extract II.....	5.44	28.2 } 25.7 } 26.9	418 } 550 } 484
10 " solution of gummy precipitate.....	5.29 5.55	22.5 } 27.9 } 25.2	54 } 44 } 49

TABLE X.

*Alcohol Extract of Wheat Germ.*

Yeast grown with addition of:	Change in $\alpha$ in 7 hrs.	Solids in moist yeast.
		<i>per cent</i>
2.5 cc. Wheat Germ Extract.....	2.08 1.86 2.15	24.5 } 24.6 } 24.5
10 " " " " .....	2.26 2.27 2.37	24.5 } 24.3 } 24.4

*Addition of Alcohol Extract to an Invertase Preparation.*

As already stated Abderhalden and Schaumann<sup>5</sup> draw the conclusion that fermentation of sugars by dried yeast or by maceration juice is also accelerated by the addition of the yeast extract. If this is true some substance in the extract must act as a coenzyme or as an activator of a proferment.

This was found not to be true, however, in the case of invertase. An invertase extract was prepared by shaking 2 gm. of fresh Fleischmann's yeast with 100 cc. of water and 2 cc. of toluene for 12 hours. Solutions were then made up in duplicate as follows:

(a) 50 cc. of a 20 per cent sucrose solution, 0.4 cc. 0.1 N HCl, 25 cc. of invertase preparation and water to make 100 cc.

(b) The same as (a) except that 5 cc. of Alcohol Extract I were substituted for an equal amount of water.

(c) The same as (a) except that 5 cc. of Benzene Extract were substituted for an equal amount of water.

These were shaken at 30°C., portions being removed at the end of 2 and 4 hours, and the rotation was noted. The results show no acceleration of inversion by addition of these solutions, one of which, the alcohol extract, was very efficacious in stimulating invertase formation as well as growth. The results are given in Table XI.

TABLE XI.

*Effect of Growth-Stimulating Substance on the Activity of the Enzyme.*

Material added:	Change in $\alpha$ in 2 hrs.	Change in $\alpha$ in 4 hrs.
5 cc. Alcohol Extract I.....	5.61	10.29
5 " " " I.....	5.75	10.60
5 " Benzene Extract.....	6.08	10.94
	6.00	10.80
5 " Water.....	5.95	10.89

This was repeated with a much less active invertase preparation with the same results. There was no possibility, therefore, that in the first case the invertase preparation itself contained sufficient of the stimulating substance to give the maximum effect.

## DISCUSSION.

It is evident from the results obtained in these experiments that some constituent of yeast is soluble in 70 to 95 per cent alcohol and still more soluble in water, which, when added in small amounts to a medium in which yeast is grown, will increase the invertase activity of such yeast to a remarkable extent. Contrary to expectation this substance seems to be something other than the growth-stimulating element. Euler considered that it was a nitrogenous substance which was easily available for yeast nutrition and which had this effect in accelerating invertase formation.

On the other hand, carbohydrates in the medium are known to cause an increase in the invertase activity of the yeast. This is especially true of mannose. Mathews and Glenn<sup>11</sup> found that active invertase preparations always contained about 70 per cent of a mannose gum. This suggests that the substance in yeast extract which has this specific effect in increasing the invertase activity of yeast is some form of carbohydrate. The fact that this activity was found in high concentration in the gummy precipitate which separated from the hot alcohol extract of yeast, is very suggestive in this connection. Further work is planned to identify the substance.

## SUMMARY.

1. The results of these experiments have confirmed the presence in a water or alcohol extract of yeast of a substance which accelerates the rate of invertase formation during a 24 hour period of growth.

2. This substance is not identical with the growth stimulant. A partial separation of the two substances has been accomplished by three methods: (a) Extraction of the growth stimulant with benzene, (b) adsorption with fullers' earth, and (c) precipitation with phosphotungstic acid.

3. The substance which accelerates invertase formation was found in high concentration in the gummy precipitate separated from an alcohol extract of yeast.

<sup>11</sup> Mathews, A. P., and Glenn, T. H., *J. Biol. Chem.*, 1911, ix, 29.

4. Extracts of wheat germ, very active in stimulating growth, did not increase the invertase concentration of yeast when added to the medium.

5. The yeast extract does not act directly upon the invertase itself. Therefore the substance does not appear to be of the nature of an activator or coenzyme.

In conclusion I wish to express to Professor F. C. Koch my appreciation of his generosity in giving helpful advice during the progress of this work.

# DETERMINATION OF THE MONOAMINO-ACIDS IN THE HYDROLYTIC CLEAVAGE PRODUCTS OF LACTALBUMIN.\*

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During recent years a large advance has been made in the methods for the determination and isolation of the amino-acids yielded by proteins on hydrolysis. The recent discovery by Dakin (1) of hydroxyglutamic acid among the hydrolysis products of casein has also been a most valuable contribution in this field. It is very probable that the lists of amino-acids determined in many proteins during the earlier years should also include this amino-acid. The only published analysis of the mono-amino-acids in lactalbumin is that published in 1907 by Abderhalden and Pribram (2). On account of the greatly increased prominence given milk as of paramount importance in human nutrition it was thought to be of interest to make a new analysis of the products of hydrolysis of lactalbumin in the light of the newer methods. In this analysis only the monoamino-acids are determined, since an analysis of the diamino-acids has been made by the Van Slyke method by Osborne, Van Slyke, Leavenworth, and Vinograd (3).

The lactalbumin used in this hydrolysis was obtained in connection with the preparation of protein-free milk used in nutrition experiments. Fresh skimmed milk was heated to a temperature of 35°C. by passing in a current of steam. Normal hydrochloric acid was then added until a test portion of the whey showed a hydrogen ion concentration of approximately 4.6. The filtrate

\* A preliminary report of this work was presented at the fifteenth annual meeting of the Society of Biological Chemists held in Chicago, December 28 to 30, 1920 (cf. *J. Biol. Chem.*, 1921, xli, p. xii).

from the precipitated casein was boiled for 10 minutes in a steam-jacketed kettle. The coagulated protein was washed several times with hot water and suspended in dilute alcohol. It was afterwards allowed to stand in absolute alcohol, then in anhydrous ether, and finally dried at 110°C. This preparation contained 5.85 per cent moisture, 0.91 per cent ash, and calculated ash- and moisture-free, 15.39 per cent of nitrogen (Kjeldahl).

#### *Hydrolysis of Lactalbumin.*

*Glutamic Acid.*—The hydrolysis was effected by boiling for 40 hours on an oil bath<sup>1</sup> 325 gm. of the lactalbumin, equivalent to 303 gm. of the ash- and moisture-free protein, with 1,450 cc. of hydrochloric acid (specific gravity 1.1). After diluting the hydrolysate somewhat, the suspended humin was removed by filtration. This amounted to 5.77 gm., and contained 7.66 per cent nitrogen. The nearly black filtrate was concentrated under reduced pressure to about 450 cc. which on cooling partly solidified. It was then redissolved in a small amount of water and saturated cold with hydrochloric acid. The crystalline product which had separated after standing at 0° for 5 days was redissolved, decolorized with norit, and resaturated with hydrochloric acid. Examination of a test portion of the glutamic acid hydrochloride thus obtained showed that it was practically free from ammonium chloride. It decomposed with effervescence at 199–200°, and gave 7.78 per cent of amino nitrogen as determined by the Van Slyke method (calculated, 7.65 per cent). There were thus isolated 29.42 gm. of glutamic acid hydrochloride equivalent to 23.57 gm. of the free acid, which, together with the 15.49 gm. subsequently obtained by the lime-alcohol method, amounts to 39.06 gm., or 12.89 per cent of the protein.

The united filtrates and washings from the glutamic acid hydrochloride were diluted with water to make a volume of about 12 liters, and hydrochloric acid was added in the amount neces-

<sup>1</sup> The material used for the oil bath consisted of a highly hydrogenated cottonseed oil, which fused at about 58–60°. We have used it for this purpose for a considerable time and have found it to be far superior to the materials commonly used for oil baths. Little or no odor or fumes are evolved when it is heated for long periods at temperatures as high as 170°.

sary to make the solution contain 3.5 per cent. The diamino-acids were then removed from the solution in the usual way by means of phosphotungstic acid and the excess of the latter removed quantitatively, first by means of ether and amyl alcohol and the remaining traces by means of barium hydroxide. The solution of monoamino-acids was treated with an excess of freshly slaked lime according to Foreman's lime-alcohol method (4) for the separation of the dibasic acids. After filtering off the excess of calcium hydroxide, the solution of calcium salts was concentrated under reduced pressure to about 1,000 to 1,200. cc. In all of the foregoing work the concentrating of solutions was done under reduced pressure and at a temperature not exceeding 40-42°, in order to avoid the formation of the pyrrolidone carboxylic acids from glutamic and hydroxyglutamic acids. The calcium salts of the dibasic acids were then precipitated in the usual way by the addition of alcohol, and the large amount of precipitate thoroughly washed with 95 per cent alcohol. This precipitate also contained considerable tyrosine, as will be shown in a subsequent paragraph. The dibasic acids were recovered by removal of the calcium with oxalic acid. The hydrochloric acid in the solution was then removed by means of silver acetate and the excess of silver with hydrogen sulfide. The solution of dibasic acids was concentrated at low temperature and, after filtering off 0.63 gm. of tyrosine, the filtrate was further concentrated until the amino-acids had separated in the form of a thick paste. This was removed from the flask as far as possible and dried *in vacuo* over powdered sodium hydroxide and calcium chloride; the parts adhering to the sides of the flask were washed out with small amounts of water and added to the main portion. The amino-acids, thus obtained and dried, weighed 87.93 gm. In order to separate the glutamic and aspartic acids from other products present the dry residue was triturated with glacial acetic acid. This residue was difficult to disintegrate with the acetic acid and formed a pasty mass which filtered very slowly. The portion remaining undissolved in the acetic acid was triturated two more times with fresh acetic acid. The disintegration of the mixture at first with acetic acid can be greatly facilitated by allowing the substance to soak in the acetic acid over night or longer and then thoroughly triturating in a mortar.



After filtering by suction the acid-insoluble substance was dried on the filter by drawing air through the suction flask and gradually reducing the lumps by means of a spatula. The nearly white powder thus obtained weighed 55.67 gm.

*Aspartic Acid*.—The above product was dissolved in about 2 liters of water and boiled with an excess of copper carbonate. After filtering off the excess of the latter the solution yielded 54.33 gm. of copper aspartate, which separated in the characteristic crystal form. This salt, after washing with cold water and drying in the air at room temperature, gave the following results on analysis:

Analysis 1. 0.3387 gm. substance: 0.0974 gm. copper oxide.

$C_4H_5C_4N\text{Cu}.4\frac{1}{2}H_2O$ . Calculated. Cu 23.07.

Found. Cu 22.98.

The filtrate from the copper aspartate on further concentration gave a pale blue copper salt, which after decomposition with hydrogen sulfide gave 2.79 gm. of pure glutamic acid. Copper was removed from the filtrate and from the copper glutamate and the concentrated solution, after saturating with hydrochloric acid, yielded 9.94 gm. of glutamic acid as the hydrochloride. The filtrate from the glutamic acid hydrochloride, after removal of hydrochloric acid by distillation and finally with silver sulfate, gave 1.96 gm. of aspartic acid in the form of the copper salt. The total amount of aspartic acid isolated was 28.18 gm., which represents 9.30 per cent of the protein.

The filtrate from the last crop of copper aspartate, after removal of the copper with hydrogen sulfide, was concentrated, and by removal of successive crops of crystals and fractional crystallization there were obtained 2.16 gm. of tyrosine and 2.76 gm. of glutamic acid. The final filtrate gave a syrup from which nothing definite could be isolated. This syrup still gave a strong positive test for tyrosine with Millon's reagent. It is of interest to note that 2.79 gm. of tyrosine, which is equivalent to 47.3 per cent of the total amount of tyrosine obtained from the protein hydrolyzed, were isolated from the dibasic acid fraction of the calcium salts which were obtained by the precipitation with alcohol.

*Hydroxyglutamic Acid*.—The acetic acid extracts remaining after triturating the product obtained from the precipitated

calcium salts were concentrated at a temperature not over 40° to a thin syrup. After standing for several weeks over calcium chloride and powdered sodium hydroxide, a thick viscous syrup was obtained. Analyses showed that it contained 2.8780 gm. of total nitrogen and 2.7115 gm. of amino nitrogen. This amount of amino nitrogen, if due entirely to hydroxyglutamic acid, represents 31.6 gm. of this acid, which is a little over 10 per cent of the protein. Subsequent careful examination of this substance indicated that the above figure represents quite closely the amount of hydroxyglutamic acid that was present in this fraction. The acid was then made nearly neutral by addition of sodium carbonate, and mercuric acetate added until no further precipitation of the white salt occurred. After thoroughly washing the precipitate with water it was decomposed with hydrogen sulfide. The filtrate from the mercuric sulfide was concentrated at 40° to about 40 to 50 cc., and a relatively large amount of absolute alcohol added. The oily product which separated was washed several times with fresh absolute alcohol. Under the treatment with absolute alcohol this product gradually hardened and was finally obtained in a coarsely granular form. It was then dried, at first *in vacuo* over calcium chloride, and finally over phosphorus pentoxide at room temperature at 16 mm. pressure. The acid thus obtained was very hygroscopic. On heating at about 60° it appeared to soften, and at about 85° it became pasty and gradually intumescend, leaving an opaque column. It did not materially change in appearance on further heating until a temperature of about 140° was reached. Between 140 and 150° it changed, with slight effervescence, to a clear liquid. This behavior on heating corresponds quite closely to that given by Dakin for hydroxyglutamic acid. A sample on analysis gave 38 per cent carbon. This figure is about 1 per cent too high for that required for hydroxyglutamic acid (36.81 per cent). This discrepancy was most likely due to the presence of a little tyrosine, as a distinctly positive test for tyrosine was obtained with Millon's reagent. On account of the high carbon content of tyrosine (59.67 per cent), only a small amount of this acid would be required to account for the above high result.

For further purification the acid was dissolved in a small amount of water, reprecipitated with alcohol, and the oil which separated

thoroughly washed with several fresh portions of alcohol. After drying, first with absolute alcohol, and finally with calcium chloride and phosphorus pentoxide *in vacuo* as above described it was analyzed with the following results:

Analysis 2. 0.2707 gm. substance: 0.3655 gm. carbon dioxide and 0.1357 gm. water.

$C_5H_9O_5N$ .	Calculated.	C 36.81, H 5.52.
	Found.	C 36.82, H 5.61.

A portion of the acid was made nearly neutral with potassium hydroxide and converted into the silver salt by alternate addition of silver nitrate and potassium hydroxide.

Analysis 3. 0.3017 gm. substance: 0.2296 gm. silver chloride.

$C_5H_7O_5NAg_2$ .	Calculated.	Ag 57.26.
	Found.	Ag 57.27.

*Alcoholic Filtrate from the Precipitated Calcium Salts.*

*Tyrosine.*—The alcoholic filtrate from the calcium salts of the dicarboxylic acids, which amounted to 5 to 6 liters was concentrated to 800 to 900 cc., and the calcium removed with ammonium oxalate. The filtrate from the calcium oxalate was concentrated and two crops of crystals were successively removed. These weighed about 8 gm., and on fractional crystallization yielded 2.89 gm. of tyrosine. This, together with the 2.79 gm., which were precipitated with the calcium salts of the dicarboxylic acids already referred to, and 0.22 gm. subsequently isolated from the barium residues of the unesterified amino-acids, amount to 5.90 gm. or 1.95 per cent of the protein. A representative sample of the tyrosine isolated gave the following results on analysis:

Analysis 4. 0.2985 gm. substance required 16.2 cc. of 0.1 N acid.

$C_9H_{11}O_3N$ .	Calculated.	N 7.73.
	Found.	N 7.62.

A complete separation of tyrosine from the other products of protein hydrolysis is extremely difficult to accomplish. This is shown by the fact that some tyrosine was either isolated, or shown to be present, by microscopic examination and tests with Millon's reagent, in most of the main fractions of this hydrolysis.

It was partly precipitated by alcohol with the calcium salts of the dicarboxylic acids, a considerable amount was obtained from the alcoholic filtrate from the calcium salts and a small amount isolated from the barium residue of the unesterified fraction. Furthermore, some was esterified and later detected in the distillation residue. Tyrosine, however, is esterified with difficulty by means of the lead salt method of esterification. Although pure tyrosine is one of the least soluble of the amino-acids obtained from proteins, nevertheless its presence has been frequently detected in residual filtrates from which such soluble amino-acids as alanine, glycine, and serine have been removed as far as possible. The percentage of tyrosine isolated as given above must therefore be regarded as minimal.

*Proline.*—The main solution of amino-acids containing the filtrates and washings from the tyrosine were concentrated to a small volume and the ammonium chloride, which had formed when removing the calcium with ammonium oxalate, was decomposed, and the ammonia expelled by boiling with a slight excess of barium hydroxide. Barium was removed and the recovered, free, dry amino-acids extracted with boiling absolute alcohol. Proline was determined according to the method described in a previous publication from this laboratory (5). Two other proline determinations made on separate hydrolysis of 10 gm. samples of the protein gave closely agreeing results. The average of all of the results obtained was 3.76 per cent.

For the separation of the amino-acids remaining after the removal of proline by extraction with alcohol, they were converted into their ethyl esters according to Foreman's method (6). For this purpose an excess of lead oxide was suspended in the solution of amino-acids and steam passed in for about 45 minutes. After filtering off and washing the excess of lead oxide the solution was evaporated to dryness, and the 216 gm. of dry lead salts obtained were suspended in absolute alcohol and the mixture was saturated at a temperature below 0° with dry hydrochloric acid gas. The greater part of the hydrochloric acid in the filtrate from the lead chloride was removed by distillation. The solution was then placed in a freezing mixture and nearly neutralized by addition of absolute alcohol which had been saturated with dry ammonia. The precipitated ammonium chloride was filtered

off and the alcohol removed by distillation under reduced pressure. The residue was dissolved in dry chloroform and the esters were liberated by means of dehydrated barium hydroxide. After removal of the excess of barium hydroxide by filtration, and the chloroform by distillation, the residual esters were dissolved in anhydrous ether and the ethereal solution was allowed to stand for several days with anhydrous sodium sulfate. There were obtained, after distilling off the ether, 149 gm. of esters.

*Glycine*.—The esters which were carried over by the vapors during the removal of the alcohol and chloroform by distillation were recovered and added to the final ether distillate from the esters. The solution was strongly acidified with alcoholic-hydrochloric acid, and allowed to stand at 0° for nearly a month. About 0.2 gm. of a white crystalline substance was filtered off. This melted to a clear oil at 138–140° indicating that it was slightly impure glycine ester hydrochloride. This was added to the residue obtained after the removal of the ether by distillation. Hydrochloric acid was quantitatively removed from the residue by means of silver sulfate, the silver with hydrogen sulfide, and the sulfuric acid with barium hydroxide. The residue remaining after evaporating the solution to dryness weighed 1.19 gm. To this was added the 0.92 gm. obtained by evaporating the final filtrate from the alanine from Fraction I of the distilled esters. From this mixture there were obtained in the usual way 2.83 gm. of glycine picrate, which decomposed at 200° (uncorrected). (Levene and Van Slyke (7) state that it softens at 199–200°, and decomposes at 202°.) The above amount of glycine picrate is equivalent to 1.12 gm. of glycine, or 0.37 per cent of the protein.

*Serine*.—The barium residues remaining after filtering the chloroform solution of the esters were decomposed with warm dilute sulfuric acid, and the barium sulfate washed until the washings gave no test for tyrosine. Hydrochloric acid was removed with silver sulfate and the sulfuric acid with barium hydroxide. The solution was then concentrated and after removal of 0.22 gm. of tyrosine the remaining amino-acids were fractionally crystallized. A fraction was obtained which consisted chiefly of serine but also contained, besides some tyrosine, a small amount of a substance which gave to the solution of the mixture a decided

acid reaction. Extensive fractional crystallization failed to effect the separation of the serine from the admixed impurities. The solution was finally neutralized by addition of a few drops of ammonium hydroxide. On slow evaporation crystals of tyrosine separated. These were removed and from the filtrate 5.34 gm. of serine crystallized in large compact plates. The serine had a decidedly sweetish taste and on heating darkened at 210–215° and decomposed with effervescence at 232–233° (uncorrected). Analysis<sup>2</sup> showed it to have the following composition:

Analysis 5. 0.1914 gm. substance: 0.2411 gm. carbon dioxide and 0.1192 gm. water.

$C_3H_7O_2N$ . Calculated. C 34.29, H 6.67.  
Found. C 34.35, H 6.97.

The esters remaining after the distillation of the ether were separated into the following fractions by distillation under reduced pressure:

TABLE I.

Fraction No.	Temperature of the bath up to.	Temperature of the vapors up to.	Pressure.	Weight.
	°C.	°C.	mm.	gm.
I.....	80	65	13	12
II.....	115	100	2	80
Distillation residue.....				16
Contents of the liquid air tube ....				22

*Fraction I. Alanine and Valine.*—To this fraction were added the contents of the liquid air tube which contained besides esters some ether and chloroform. After hydrolyzing the esters by boiling with water for 7 to 8 hours, the solution was concentrated under reduced pressure and six crops of crystals were successively removed. Absolute alcohol was added to the filtrate from the last crop and the precipitated amino-acids filtered off. The final filtrate was evaporated to dryness and the residue together with the crop which was precipitated by alcohol added to the amino-acids which were examined for glycine. By fractional crystallization from water and from water and alcohol

<sup>2</sup> The carbon and hydrogen determinations recorded in this hydrolysis were made by Mr. C. E. F. Gersdorff.

there were obtained 7.29 gm. of alanine, and a small amount of a mixture of leucine and valine from which were isolated, by means of the lead salt method of Levene and Van Slyke (8), 1.37 gm. of leucine and 1.37 gm. of valine. Analysis of the alanine gave the following results.

Analysis 6. 0.1612 gm. substance: 0.2373 gm. carbon dioxide and 0.1161 gm. water.

$C_3H_7O_2N$ .	Calculated.	C 40.45, H 7.86.
	Found.	C 40.15, H 8.06.

*Fraction II. Leucine and Valine.*—After hydrolysis of the esters of this fraction in the usual way the aqueous solution of amino-acids was concentrated under reduced pressure, and eleven crops of amino-acids were obtained by their successive removal as they separated out in the distillation flask. The first three crops weighing 28.11 gm. were practically pure leucine and 1.38 gm. were further obtained by fractional crystallization of the fourth crop.

Analysis 7. 0.1813 gm. substance: 0.3657 gm. carbon dioxide and 0.1565 gm. water.

$C_6H_{13}O_2N$ .	Calculated.	C 54.96, H 9.99.
	Found.	C 55.01, H 9.66.

Further fractional crystallization and carbon and hydrogen determinations showed that the remaining acids of this fraction consisted of considerable valine. Use was made of the lead salt method of separation and 17.61 gm. of the lead salt of leucine (equivalent to 9.88 gm. of leucine) were isolated. The free leucine obtained by decomposition of the lead salt with hydrogen sulfide gave on analysis 54.91 per cent carbon, and 9.96 per cent hydrogen.

The amino-acids remaining in the filtrate from the lead leucine were recovered in the usual way. Difficulty was encountered in isolating the valine in a pure form on account of the presence of some substance which had strong acidic properties. Subjection of the mixture to the lime-alcohol method for removing the dicarboxylic acids proved to be inadequate for the complete removal of this acid, although a very small amount of a syrup was obtained from the calcium salts precipitated by the alcohol.

This syrup was insoluble in alcohol, strongly acidic, and could not be brought to crystallization. On heating with zinc dust it gave a strong pyrrole test. Lack of material prevented any further examination of this substance. The only explanation which we can advance at present as to its character and presence in the leucine fraction is that not all of the hydroxyglutamic acid was precipitated together with the other dicarboxylic acids as the calcium salts, and that this portion which escaped precipitation was subsequently esterified and that some of its ester distilled over with the leucine and valine esters of Fraction II.

The mixture of amino-acids was recovered from their calcium salts and recrystallized from water, after having first made the solution faintly alkaline with ammonium hydroxide. In this way most of the crystalline amino-acids were obtained free from the acid impurity, and after removal of 1.73 gm. of leucine as the lead salt 8.63 gm. of fairly pure valine were obtained.

Analysis 8. 0.1885 gm. substance: 0.3549 gm. carbon dioxide and 0.1586 gm. water. 0.1400 gm. substance required 11.8 cc. of 0.1 *N* sulfuric acid.

$C_5H_{11}O_2N$ .	Calculated.	C 51.28, H 9.47, N 11.96.
	Found.	C 51.35, H 9.42, N 11.83.

*Phenylalanine*.—The residue remaining after the distillation of the esters was shaken with water and ether in the usual way in order to remove the phenylalanine ester. The ester extracted by the ether was dissolved in concentrated hydrochloric acid and the solution evaporated to 15 to 20 cc. on a steam bath. From this solution there were obtained 3.75 gm. of phenylalanine in the form of the hydrochloride. The free phenylalanine obtained by decomposition of the hydrochloride with ammonium hydroxide gave the following results on analysis:

Analysis 9. 0.1421 gm. substance: 0.3418 gm. carbon dioxide and 0.0838 gm. water.

$C_9H_{11}O_2N$ .	Calculated.	C 65.45, H 6.66.
	Found.	C 65.60, H 6.60.

The aqueous solution remaining after removing the phenylalanine ester with ether was boiled with barium hydroxide and the barium quantitatively removed with sulfuric acid. Aside from a small amount of crystals, which both from appearance and



composition indicated leucine, nothing of a definite character could be isolated.

# DISCUSSION.

The results of the preceding analysis of the products formed by the hydrolysis of lactalbumin are collected in Table II, together with those obtained by Abderhalden and Přibram. The percentages of the diamino-acids obtained by Osborne, Van Slyke, Leavenworth, and Vinograd, as determined by the Van Slyke method, are also given and added to those of the monoamino-acids determined in this analysis. In comparing our results with those of Abderhalden and Přibram the most striking differ-

TABLE II.  
*Percentage of Amino-Acids in Lactalbumin.*

Amino-acid.	Jones and Johns	Abderhalden and Přibram
Glycine.....	0.37	
Alanine.....	2.41	2.5
Valine.....	3.30	0.9
Leucine.....	14.03	19.4
Proline.....	3.76	4.0
Phenylalanine.....	1.25	2.4
Aspartic acid.....	9.30	1.0
Glutamic acid.....	12.89	10.1
Hydroxyglutamic acid.....	10.00	
Serine.....	1.76	
Tyrosine.....	1.95	0.85
Total monoamino-acids.....	61.02	41.15
Cystine.....	1.73*	
Arginine.....	3.47*	
Histidine.....	2.61*	
Lysine.....	9.87*	
Tryptophane.....	Present.	
Ammonia.....	1.31*	
Total diamino-acids and ammonia.....	18.99	18.99*
Total.....	80.01	60.14

\* Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 266.

ence, aside from the hydroxyglutamic acid, is shown by the figures for aspartic acid, the percentage of this amino-acid found in this hydrolysis being over nine times that previously recorded. It is generally recognized by investigators who are familiar with the details of the analysis of the products of protein hydrolysis that the determination of aspartic acid as made by the ester method of Emil Fischer is very uncertain. Osborne and Jones (9) in a study of the consideration of the sources of loss in analyzing the products of protein hydrolysis were able to recover only 42.5 per cent of the aspartic acid which was used in a mixture of amino-acids after these amino-acids had been subjected to the same treatment as employed in the hydrolysis of proteins and the analysis of their degradation products by means of the Fischer method. The difficulty in the way of obtaining better results in the determination of aspartic acid by the Fischer method has been due to several causes: Incomplete esterification, losses through decomposition of the esters during distillation, and incomplete saponification with the formation of the half ester. The chief cause, however, has been undoubtedly due to the fact that the aspartic acid was often associated with considerable quantities of pyrrolidone carboxylic and hydroxyglutamic acids. These acids form syrupy mixtures from which the aspartic acid would crystallize very slowly if at all; the same would correspondingly apply to their copper salts. By means of the newer methods used in this hydrolysis these difficulties have been entirely avoided. The new result for valine is considerably higher, while the difference in the combined values for valine and leucine is not great. This higher figure for valine is probably due to a more complete separation of this acid from the leucine, which was made possible by means of the lead salt method of Levene and Van Slyke (8). The percentages of alanine, proline, and glutamic acid are in fairly close agreement. Our figure for tyrosine, although twice that found by Abderhalden and P'ibram, is without question too low, since evidences of tyrosine were found in several fractions, from which it could not be isolated in pure enough condition to be weighed. The considerable amount of hydroxyglutamic acid found is of interest, especially so inasmuch as Dakin (1) first discovered this amino-

acid in about the same percentage in casein. This makes the total proteins of milk particularly high in this most recently discovered amino-acid constituent of proteins.

#### SUMMARY.

The lactalbumin was prepared from fresh skim milk. The casein was first precipitated at 35° by normal hydrochloric acid at a hydrogen ion concentration of 4.6. The lactalbumin obtained by boiling the filtrate from the casein for 10 minutes, was dried with alcohol and ether, and finally at 110°.

The protein was hydrolyzed by boiling for 40 hours with hydrochloric acid (specific gravity 1.1) and the resulting monoamino-acids determined, use being made of the more recent methods for their separation.

The outstanding results of this analysis consists in the isolation of 0.37 per cent of glycine, 1.76 per cent of serine, and at least 10 per cent of hydroxyglutamic acid. These amino-acids have not been heretofore determined in the hydrolysis products of lactalbumin. A yield of 9.30 per cent of aspartic acid was obtained, which is nine times the amount previously recorded. These percentages, together with those of the other monoamino-acids, are tabulated and compared with the recorded results of a previous hydrolysis of lactalbumin.

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# THE ZINC AND COPPER CONTENT OF THE HUMAN BRAIN.

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The presence of traces of copper in the human brain was first reported by Thudichum (1). In commenting on Thudichum's work Mathews (2) suggests that this point should be reinvestigated to see whether copper is in reality a normal constituent of all brains. He considers the possibility that the human brains which Thudichum examined might have come from brass-workers or others exposed to copper poisoning.

Despite the statement of Palet (3) who was unable to detect copper in 54 normal human livers there is general agreement among investigators that copper as well as zinc are normal constituents of plant and animal tissues. At the time the analyses recorded in this paper were begun no data concerning the occurrence of zinc in the human brain had appeared in the literature. Recently Rost (4) reported an analysis of a human brain containing 11 mg. of zinc per kilo.

Copper and zinc have been shown to be widely distributed in foods. The continuous ingestion of these metals raises the question as to the extent of their storage in various organs. The relative tolerance of zinc and copper by the animal organism, especially when introduced with foods has been noted by a number of observers. In a brief study of the fate of zinc in the animal organism, Salant, Rieger, and Treuthardt (5) found that after intravenous injection of zinc malate in cats the metal was stored in considerable amounts in the liver, that it was almost always found in the skin and muscles but that none was present in the brain. Giaya (6), on the other hand, finds that the partition of zinc per organ occurs in the following decreasing order: brain, lungs, stomach, liver, kidneys, intestines, heart, spleen.

*The Zinc and Copper Content of the Human Brain.\**

Case No.	Race or nationality.	Age.	Occupation.	Weight of brain gm.	Amount taken for analysis	Zinc.			Copper.			Cause of death.
						Found in sample.	In entire brain.	In 1,000 gm. of brain.	Found in sample.	In entire brain.	In 1,000 gm. of brain.	
						mg.	mg.	mg.	mg.	mg.	mg.	
1	Norwegian.	21	Seaman.	1,630	100	0.52	8.48	5.2	0.36	5.87	3.6	Fractured skull.
2	Negro.	23	Laborer.	1,157	100	0.88	10.18	8.8	0.60	6.94	6.0	Acute dilatation of the heart.
3	"	35	"	100	100	0.72		7.2	0.47		4.7	Angina pectoris.
4	Mexican.	40	"	100†	100†	1.57		15.7	0.60		6.0	Lobar pneumonia.
5	White.	Fetus about 5 months.		64.5	64.5	0.74	0.74	11.5	0.44	0.44	6.8	Nothing to suggest disease of fetus.

\* The brains and autopsy records were kindly furnished us by Dr. H. C. Hartman and Dr. Anna M. Bowie of the Pathology Department.

† Analysis of the cerebrum. A separate analysis of the cerebellum of this brain gave 17.5 and 6.7 mg. per kilo of zinc and copper respectively.

Believing the subject to merit further attention we are presenting the results of the analyses of four adult brains and of a fetal brain. These were received in the laboratory immediately after autopsy, washed free of blood with physiological salt solution, and analyzed according to the methods previously described by Pose and Bodansky (7) and Bodansky (8, 9).

The results indicate that copper and zinc occur normally in the human brain, there being nothing in the records of any of the individuals to suggest exposure to zinc or copper poisoning. It will be observed that the values for copper fall within the limited range of 3.6 and 6.8 mg. per kilo. The proportion of zinc in the fetal brain was found to be greater than in three of the adult brains, the proportion of copper being greater than in any of the adult brains. A number of similar observations by other investigators may be mentioned. Ghigliotto (10) analyzed the viscera of a 7 months' old fetus and found the proportion of zinc to be slightly higher than in adults. Giaya (6) found 3 mg. of zinc in 100 gm. of a fetus weighing 420 gm. It appears that during intrauterine life there is more rapid accumulation of zinc and copper as well as of other inorganic constituents, than there is after birth. According to Fenger (11) the thyroids of beef fetuses contain more iodine and phosphorus per unit of body weight than thyroids from fully mature animals. The brain of a newly born albino rat contains greater proportions of phosphorus and sulfur than does the brain of an adult rat, according to the analyses of M. L. Koch (12). That there is a decrease in the ash content of the human brain with growth has been shown by W. Koch and Mann (13). In this connection it may also be of interest to recall that Maquenne and Demoussy (14) recently found in their studies on the migration of copper in the tissues of green plants that copper is most abundant in young actively growing tissues.

#### SUMMARY.

The results of the analyses of four adult brains and of a fetal brain indicate that copper and zinc are normal constituents of the human brain. Judging from our analysis of the one fetal brain, it appears that during intrauterine life there is more rapid storage of zinc and copper in the brain than there is after birth.

In this respect the behavior of these elements is similar to that of other inorganic constituents of animal tissues such as iodine, sulfur, and phosphorus.

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## A SIMPLIFIED FORM OF APPARATUS FOR AIR ANALYSIS.

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(Received for publication, July 22, 1921.)

The apparatus was designed primarily for general student use, therefore, simplicity and cost were considerations as well as reasonable accuracy. In service it has proved so satisfactory in general that it is described for the possible benefit of others. It differs from the well known forms in dimensions rather than in principles or in design.

It consists of a gas burette (Fig. 1, A) of bulb and stem form having a total calibrated capacity of 40 cc. From the tap to the upper calibration of the stem is 30 cc. The stem is calibrated from 30 to 40 cc. in 0.1 cc.

A water jacket of glass surrounds the bulb and stem of the burette (Fig. 1, B). It terminates below in a short neck. The internal diameter is about 8 mm. greater than the external diameter of the stem of the burette. The two stems are fixed and sealed together with a rubber connector at a point below the 40 cc. mark on the stem of the burette. The capacity of the jacket is about 90 cc. The totalunjacketed air space is about 1.5 cc. or 3 to 4 per cent. Three narrow cork wedges inserted between the outer wall of the bulb of the burette and inner surface of the expanded portion of the water jacket together with the rubber connector on the stem below firmly unites them. The stem of the jacket is securely attached to the base by two nicked spring clamps.

From the two-way tap, rise two short tubes bent at right angles. These tubes and all other glass tubes connecting the burette with the absorbers, including the stems of the latter, are thick walled and have narrow bores (about 2 mm.). The ends



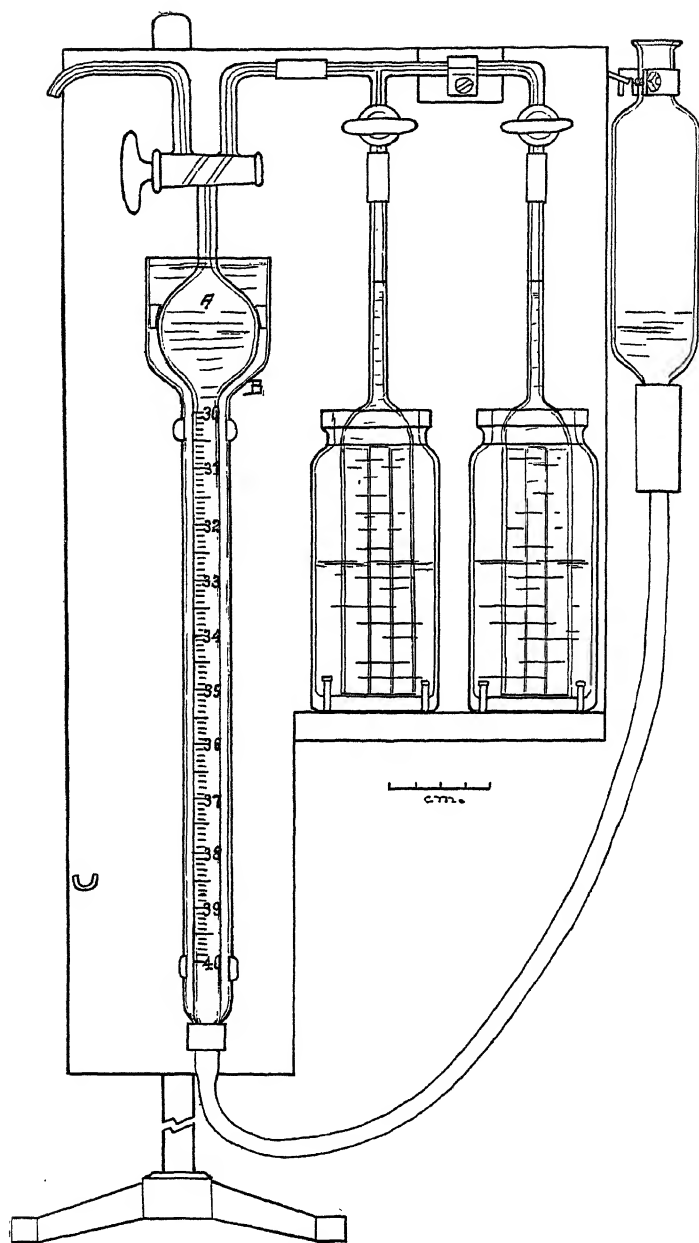


FIG. 1. Oxygen and carbon dioxide analyzer for expired air.

are slightly tapered to facilitate union by small bore, thick walled, pure rubber connectors. One of the bent tubes is for filling the burette and the other for connecting it with the tube leading to the absorbers. The free end of the filling tube is bent downward slightly in order that any displacing liquid forced through it will accumulate on the end, from which any surplus is conveniently removed by a sponge of absorbent material.

The tube leading to the absorbers consists of a horizontal part, a T and an inverted L limb. Near the middle of and integral with each of these limbs is a small one-way glass tap. The free horizontal end is attached to the burette by a short rubber connector. It is supported by a wood block, into a groove of which it is pressed by a nicked brass clamp fastened by a screw.

The absorbers are of tubular type and identical in construction. A horizontal mark or volume indicator is etched in the middle of the upper division or stem. The absorbers are attached to the T and L limbs by rubber connectors. The upper end of the absorber is dome-shaped and the stem rises from the center. The lower end is open and rests on the bottom of the absorber jacket which is an ordinary wide mouth 6 oz. bottle. The wall of the absorber is of ordinary thickness. The greatest external diameter is limited to 29 mm. which is slightly less than the internal diameter of the narrowest part of the bottle neck. To increase the absorbing surface, the absorber chamber is filled with a bundle of medium sized, thin walled, glass tubing, of such length that the upper ends extend to near the shoulders of the absorber when the lower ends are flush with the lower end of the absorber. When assembled and mounted, these tubes stand upon the bottom of the absorber bottles. Tubing of fair size is best for this purpose as it is efficient and gas bubbles do not stick among the tubes as may happen with slender tubing, particularly when very concentrated oxygen absorbing solutions are used. The absorber bottles are supported on a shelf which is provided with spring holders. These securely fix the bottles so that when assembled the apparatus can be moved about freely or transported. The holders are two slender brads driven perpendicularly part way into the shelf in such position that they press the bottles firmly against the front of the base behind the shelf.

The filling and displacing reservoir is an elongated, parallel sided glass bulb, having a capacity of 100 cc. The lower end terminates in a short stem, shaped for connecting with the rubber tube which leads to the gas burette. This tube has an external diameter of 8 mm. and wall of medium thickness. It is of pure rubber and is 45 cm. long. A thick rubber collar slipped over the connection with the stem of the reservoir provides a comfortable grip for handling it and guards against the temperature of the hand materially affecting the solution. The upper end of the reservoir is finished with a small neck with flaring mouth. To the neck is attached a metal hook for hanging the reservoir from staples driven part way into the base for the purpose. The hook is of wire, twisted about the neck and rendered immobile by winding with a strip of adhesive tape. A special nickeled hook with a clamp fastening with a screw is neater.

The base is 43.5 cm. long, 21.5 cm. wide, and 1.2 cm. thick. It is of soft wood, finished and painted black. Attached firmly to the back near the top and perpendicular to the surface, is a short nickeled rod for attaching by a clamp to a tripod stand. To the front, a wooden shelf is attached for supporting the absorbers and a wooden block for holding the glass tube connecting the burette and absorbers. The spring water jacket clamps are attached to the base with screws. The staples for suspending the displacing reservoir are driven part way into the base. One is near the top of each of the outer edges and another in the face near the left edge at about the level of the under surface of the absorber shelf.

#### *Solutions Employed.*

Distilled water containing 0.5 per cent (or less) of sulfuric acid and a little coloring matter (Orange G is satisfactory) is used in the reservoir for controlling the gas in the apparatus, about 75 cc. being a suitable amount. Owing to the comparatively short length of the gas burette, drainage is rapid, 3 minutes being the usual time allowed, even when measuring 40 cc. of gas. The pigment in the solution gives a visible index of drainage and also facilitates reading of the column of liquid on the graduation marks. A white strip of adhesive tape or paper pasted on the front of the support behind the stem of the burette further facilitates reading.

For absorbing carbon dioxide, 10 per cent aqueous sodium hydroxide solution is used, 100 cc. being introduced into the bottle of the absorber nearest the gas burette.

For absorbing oxygen an aqueous solution of potassium hydroxide and pyrogalllic acid, as recommended by Haldane<sup>1</sup> is used. Some samples of potassium hydroxide after the addition of the pyrogalllic acid, have given solutions of a grumous consistency in which case the addition of a little distilled water has rendered them satisfactory.

A layer (5 cc.) of paraffin oil is placed on top of the solution in the oxygen absorber to protect the solution from outside air. Analyzers set up with such solutions more than 6 months and used regularly and receiving no attention other than keeping the water jackets filled, show no deterioration in absorptive activity.

#### *Comments.*

For making atmospheric or expired air analyses, allowing 3 minutes for drainage yields good results. After reading the initial volume of air to be analyzed, it is passed five times into the carbon dioxide absorber, and after drainage, a new volume reading is taken. The air is then passed into the oxygen absorber ten times, then into the carbon dioxide absorber three times, to remove oxygen from the connections, and then back into the oxygen absorber ten more times and after drainage the final volume reading is taken. Of course a trace of oxygen will be left in the carbon dioxide absorber connections but if the oxygen absorber is in good condition the amount will be beyond the limits of the instrument to detect. If doubt exists, it is very easy to pass the air back into the carbon dioxide absorber again to control this point.

In displacing the air from the burette into the absorbers, care is taken to avoid entrance of the displacing liquid into the upper stem or tap of the burette.

The time required for carbon dioxide analysis, after the air is taken into the burette, is about 7 minutes and for a carbon dioxide and oxygen analysis, about 15 minutes. Perhaps it is trite to remark that such analyses should be undertaken only under suitable conditions, as uniform temperature of the liquids in the apparatus, room temperature, and absence of drafts.

<sup>1</sup> Haldane, J. S., *Methods of air analysis*, London, 2nd edition, 1918, 12, 13.

As Haldane points out the best practical control of such an instrument is the analysis of atmospheric air. With reasonable practice, consistent results, closely approximating the theoretical, are obtained for atmospheric oxygen.

TABLE I.  
*Results of Room Air Analyses.*

Date.	Carbon-dioxide.	Oxygen.	Test No.	Operator.	Apparatus No
1921	<i>per cent</i>	<i>per cent</i>			
Jan. 28	0	20.875	1	A	I
" 28	0	21.00	2	"	I
" 28	0	20.89	3	"	I
" 28	0	21.00	4	"	I
" 28	0	20.875	5	"	I
Mar. 9	0.05	20.95	1	B	II
" 9	0.05	20.95	2	"	II
" 9	0.05	20.95	3	"	II
" 9	0.05	20.925	1	"	I
" 11	0	20.95	1	A	VII
" 11	0.05	20.975	2	"	VII
" 11	0.05	20.975	3	"	VII

TABLE II.  
*Successive Analyses of Expired Air Taken from an 80 Liter Volume Held in a Bell Form of Spirometer.*

Time.	CO <sub>2</sub>	O <sub>2</sub>	Total.	Operator.	Apparatus No.
After 2 minutes.	4.00	16.43	20.43	B	I
" 7 "	4.00	16.45	20.45	A	VII
" 56 "	3.99	16.46	20.45	"	VII
" 62 "	4.02	16.33	20.35	B	I
" 302 "	3.98	16.43	20.41	A	VII
" 383 "	3.98	16.33	20.31	B	I

In view of its simplicity, compactness, portability, reliability, and speed of operation, the apparatus is well adapted for purposes permitting the limits of accuracy stated, as ordinary student or clinical use and certain types of experimental studies.

It permits of satisfactory performance of much work that otherwise would not be undertaken, owing to considerations such as the cost of standard forms, the time required to keep them in order

and to become proficient in their use and to conduct analyses. The glass parts were purchased at a cost of \$11.00. The burettes were calibrated and mounted in our shop.

### *Results.*

The results presented were selected to approximate average character and were obtained under ordinary conditions, so no doubt with greater refinement of technique, such as reading the scale with a lens, greater accuracy is possible. The skill of the operator, particularly in reading the burette is all important for minute accuracy as 0.01 cc. is estimated on the scale.

### SUMMARY.

A simplified form of analyzer for expired air is described which is well adapted for the use for which it was designed; *viz.*, general student, clinical, and certain types of experimental work.



## A GAS RECEIVER OF CONVENIENT AND PRACTICAL FORM FOR SAMPLING EXPIRED AIR FOR ANALYSIS.

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(Received for publication, July 22, 1921.)

In air analysis, perhaps the ideal technique is to take the sample directly into the gas burette, thus avoiding possibility of change in composition. Sometimes, however, it is desirable to collect and hold samples for subsequent analysis. The mercury receiver gives excellent results for collecting and holding samples, but for purposes where maximum accuracy is not essential, a less expensive and simpler method was desired. To meet this need, the method herein described, was developed.

A glass bottle having a capacity of 500 cc. and narrow, ground neck is employed as a receiver (Fig. 1). The bottle is provided with a two-holed rubber stopper, fitted with straight glass tubes of 5 mm. external diameter. The outer ends project about 1 cm. and over each of these is slipped the end of a 10 inch length of tight fitting, pure rubber tubing. Spring clamps are used for closing the rubber tubes. The inner end of one of the glass tubes terminates at the inner surface of the stopper, while the inner end of the other almost touches the bottom of the bottle when the stopper is firmly inserted into the neck. The holder consists of an ordinary rod stand about 65 cm. tall, fitted with two support rings, and a burette clamp adjusted to a single-hole stopper through which the end of an 8 cm. narrow stemmed glass funnel is thrust. The burette clamp is fastened near the top of the rod stand. The opening in the upper ring support is slightly larger than the widest diameter of the bottle and is fastened a little below the top clamp. The lower ring is less than the shoulder diameter of the bottle but greater than that of the neck and is placed about 5 to 10 cm. below the larger ring. The lower ring



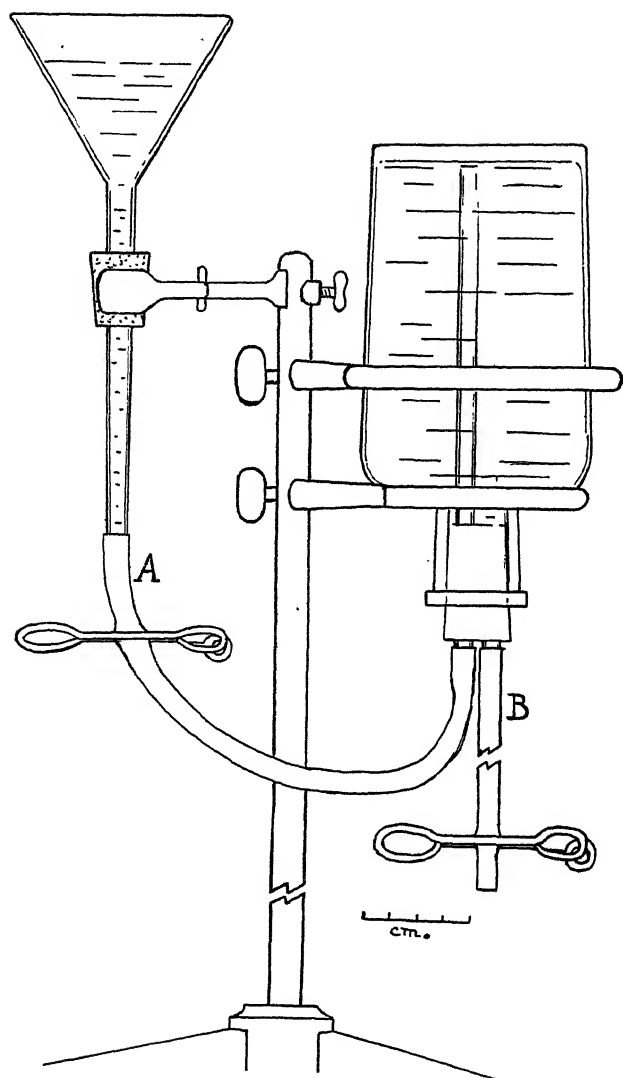


FIG. 1. Air sample receiver, Form 1. As shown it is filled with water, inverted, and is ready for disconnecting the funnel and filling with an air sample.

serves to support the weight of the bottle in either the upright or inverted position, the neck with stopper and rubber tubes extending through and below it in the latter position, while the upper ring maintains it perpendicularly and holds it on the lower one. Thus the upper ring serves as a guide while the lower one serves as a support or rest.

The bottle is filled by placing it in the upright position, attaching the stem of the funnel to the rubber tube connected with the glass tube that extends to near the bottom of the bottle, and pouring liquid into the funnel, the other rubber tube being open to permit the air in the bottle to escape. Water, to which a little phenol-sulfonephthalein has been added, is used for a filling and displacing liquid. When the bottle is almost filled, the funnel is disconnected and residual air from the lungs forced through the liquid in the bottle until the tint of the indicator becomes stabilized. The funnel is then reconnected and water from a flask, similarly saturated with expired air, is added until the bottle and tubes are full. The outer ends of the tubes are then clamped.

Using a funnel as described gives good results, the water used being saturated with expired air in a flask and poured into the funnel immediately before it is to run into the receiver. Conditions are favorable for loss of absorbed gas, however, owing to exposure to room air in pouring and also to the relatively large surface exposed in the funnel. Even so, considerable time of exposure in a funnel is required to produce a marked change in the carbon dioxide content and results show that but little error is introduced.

To reduce the objectionable features of the funnel and to render the technique more convenient and time-saving, a flask provided with a connection in the center of the bottom for the outflow tube is substituted for the funnel (Fig. 2). The flask is fitted with a stopper having three holes. Into one of these holes is fitted a short stemmed thistle tube, the lower end of which terminates just below the inner surface of the stopper. This is used for filling. One of the holes through the stopper is left open to provide a vent for the air in filling. Into the other hole is fitted a glass tube which extends to near the bottom of the flask. Its outer end is short and is bent to a right angle. To this is connected a rubber tube about 12 cm. long which serves as a connection for blowing expired air through the water in the flask, thus saturating

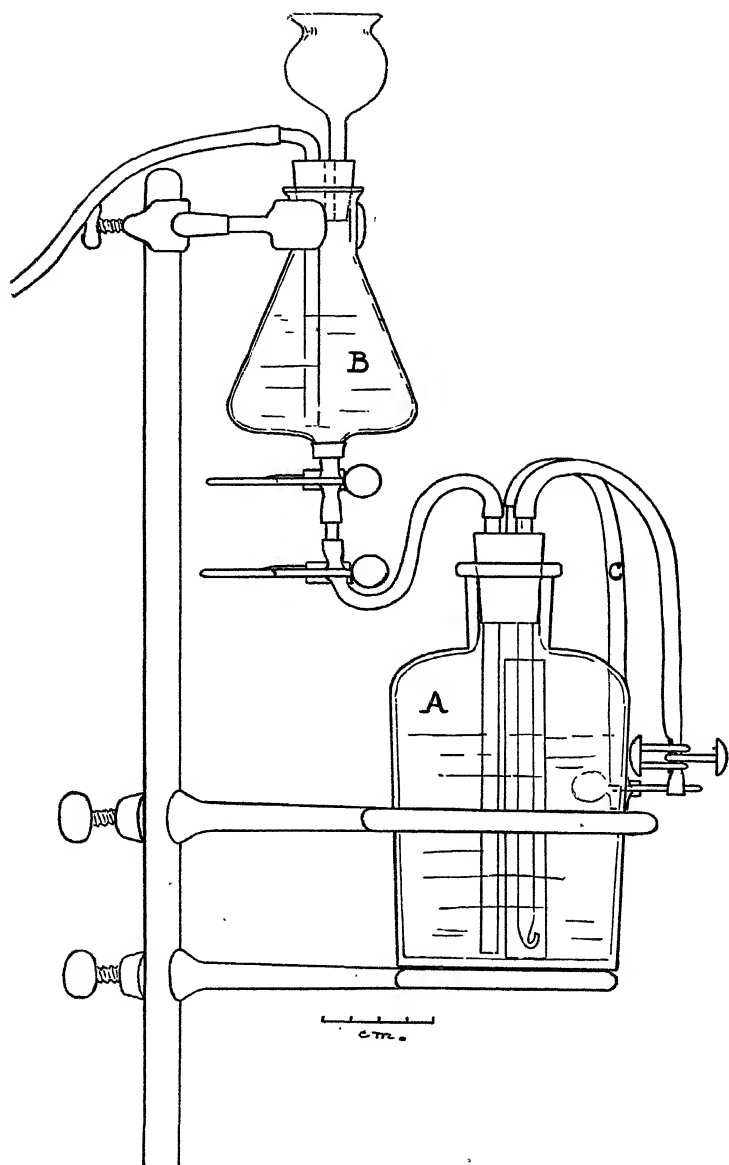


FIG. 2. Air sample receiver, Form II. Air sample receiver, *A*, with displacing reservoir, *B*. The tube *C* connects to the gas burette.<sup>1</sup>

<sup>1</sup> The receiver is a form designed for continuously sampling expired air in its passage through a mixing chamber. (For full description and method see article in the *Journal of Laboratory and Clinical Medicine*, now in press.)

and keeping it in this state. Owing to the almost closed condition of the flask, and the relatively small area of water exposed to the air as compared to the funnel, loss of gases from the water saturated in the flask type of reservoir is slower.

To collect a sample, the funnel or flask is disconnected and the bottle inverted (Fig. 1). The end of the filling tube is connected with the air reservoir to be sampled (avoiding, of course, any air in the connections other than air that is to be sampled), and the bottle filled by opening the rubber tube which acts as a siphon. As the water escapes, the air is drawn into the bottle, being delivered above the surface of the water by the long inside tube. When the bottle is filled with the air sample, both rubber tubes are clamped near the ends as before and disconnected from the spirometer.

When ready to analyze, the bottle is placed in the upright position and the rubber tube connected with the long glass tube, *i.e.* the filling tube, attached to the displacing reservoir (Fig 2). The reservoir is filled with water saturated with expired air from a flask that is kept stoppered excepting when removing its contents. Any air bubbles in the connections are removed by compressing the rubber tube or inserting a piece of wire. The clamp on the tube connected with the reservoir is then opened and water enters the bottle until the air is compressed and stops the flow. Care is exercised to keep the reservoir well filled to avoid the entrance of air. Water in the other rubber tube and connection is expelled by cautiously opening the clamp upon it with the tube in the dependent position. If care is not exercised in this operation, too much air is suddenly released, the reservoir empties too rapidly, and air enters the bottle.

The end of the tube is now ready for connection with the filling tube of the gas burette or analyzer. As the connection is made, a little air is permitted to escape from the receiver by slightly opening the clamp, in order that all other air in the connection may be displaced. As air passes into the analyzer, the pressure in the receiver falls and water enters from the reservoir.

#### *Comments.*

The loss of gas is accelerated after the introduction of water into the receiver. If a series of tests upon a single sample is desired the loss may be retarded by the introduction of a few cubic centi-

## 378 Gas Receiver for Sampling Expired Air

meters of paraffin oil into the receiver but this is objectionable owing to the action of the oil on the rubber parts.

Acidulating the water used in the receiver with sulfuric acid perhaps would be more satisfactory for this purpose.

### Results.

Results indicating the variations ordinarily encountered are shown in Table I.

TABLE I.

(a) Results of successive analyses of expired air taken directly into gas burettes from a spirometer containing 80 liters; (b) samples of the same air taken into receivers and allowed to stand for varying periods before analysis; (c) results from receivers showing the acceleration of loss of carbon dioxide after the introduction of water.

It should be noted that the figures for oxygen given in the table are not corrected for shrinkage of air volume, due to absorption of carbon dioxide. When thus corrected the actual change in oxygen content is very small.

	CO <sub>2</sub>	O <sub>2</sub>	Total.	Observer.	Instrument No.
<b>Spirometer.</b>					
After 7 minutes.....	4.00	16.45	20.45	A	VII
" 2 " .....	4.00	16.43	20.43	B	I
" 56 " .....	3.99	16.46	20.45	A	VII
" 62 " .....	4.02	16.33	20.35	B	I
" 302 " .....	3.98	16.43	20.41	A	VII
" 383 " .....	3.98	16.33	20.31	B	I
<b>Receiver I.</b>					
After 34 minutes.....	3.95	16.50	20.45	A	VII
" 38 " .....	3.95	16.38	20.33	B	I
" 74 " .....	3.95	16.50	20.45	A	VII
" 78 " .....	3.93	16.40	20.33	B	I
" 253 " .....	3.63	16.60	20.23	A	VII
" 253 " .....	3.65	16.67	20.32	B	I
<b>Receiver II.</b>					
After 275 minutes.....	3.98	16.47	20.45	A	VII
" 279 " .....	3.93	16.43	20.36	B	I
" 317 " .....	3.95	16.53	20.48	A	VII
" 302 " .....	3.98	16.40	20.38	B	I
" 322 " .....	3.93	16.43	20.36	"	I

### SUMMARY.

A simple method is described for collecting and storing samples of expired air for analyses which yields results having an error of about 1 per cent of carbon dioxide and considerably less than this for oxygen for periods of some hours.

## BACTERIA AS A SOURCE OF THE WATER-SOLUBLE B VITAMINE.

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(Received for publication, August 10, 1921.)

In the literature dealing with the vitamine content of various substances there have been several allusions to the possible presence of vitamine in cultures of bacteria. Pacini and Russell (1) apparently demonstrated the presence of a growth-promoting substance in extracts of cultures of *Bacillus typhosus* which were fed to white rats that were limited to a diet devoid of water-soluble B. Thjötta (2) has shown that a favorable influence is exerted upon the growth of *Bacillus influenzae* by the addition of sterile bacterial extracts to the broth in which the organism is planted and he suggests that this may be due to the presence in the extracts of "substances belonging to the class of the so called vitamins." On the other hand Cooper (3) has found that extracts of *Bacillus coli* had no effect in relieving polyneuritis in pigeons.

It will be noted that in each of the cases cited above a different standard is employed by which the presence or absence of the vitamine is determined. The only factor that is common to all is the kind of vitamine that is under consideration, which in each case is the water-soluble, growth-promoting vitamine provided we assume the identity of the growth-promoting and antineuritic substances.

In this paper it will be shown that certain bacteria, at least, do not produce the growth-promoting substance known as water-soluble B. The organisms tested for this vitamine were *Bacillus paratyphosus* B, *Bacillus coli*, *Bacillus subtilis*, and whatever other organisms there are that make up the intestinal flora of the white rat when this animal is limited to a diet of known composition. The criterion employed to judge of the presence of vitamine was the weight curve of the growing white rat.

*Technique.*

An attempt was made to repeat the work of Pacini and Russell but all efforts to obtain a profuse growth of *Bacillus typhosus* on the same medium that they used, *i.e.* Ushinsky's medium, were unsuccessful. It was found possible, however, to get good growths of *Bacillus paratyphosus* B and *Bacillus coli*, and as these organisms all belong to the same general group they were substituted for *Bacillus typhosus* in this work. In the third experiment it was desired to add large quantities of one of the common forms of bacteria to the ration, so *Bacillus subtilis* was chosen and in this case plain nutrient agar was used as the culture medium.

The administration of the bacteria was carried out in the following way. The organisms were grown in flasks containing 100 cc. of the medium, then killed by autoclaving at 120° for 15 minutes—a procedure that would not effect the vitamine content as has been demonstrated by McCollum, Simmonds, and Pitz (4). The culture was then concentrated by evaporation on the steam bath to about 15 to 20 cc. and finally the organisms were taken up on starch by desiccation in a shallow pan at a reduced pressure. This starch bearing the bacteria was then used to replace an equivalent amount in the basal ration and the effect of bacteria noted by observing the trend of the weight curve.

The composition of the culture medium was:

	<i>gm.</i>
Asparagine.....	3.4
Ammonium lactate.....	10.0
Sodium chloride.....	5.0
Magnesium sulfate.....	0.2
Calcium chloride.....	0.1
Acid sodium phosphate.....	1.0

The basal ration used in all the experiments had the following composition:

	<i>gm.</i>
Casein.....	18.0
Starch.....	32.5
Sugar.....	17.0
Lard.....	15.0
Butter fat.....	10.0
Salt mixture.....	2.5

The salt mixture is that used by Osborne and Mendel (5) and has the following composition:

	gm.
Calcium phosphate.....	10.0
Acid potassium phosphate.....	37.0
Sodium chloride.....	20.0
Sodium citrate.....	15.0
Magnesium citrate.....	8.0
Iron citrate.....	2.0
Calcium lactate.....	8.0

The ingredients of the basal ration were mixed together in a mortar, the starch bearing the bacteria was added at this time, and the resulting paste was fed to the rats in small glass dishes.

By inspection it will be seen that the above ration is adequate in all dietary essentials with the exception of the water-soluble vitamine and it has been demonstrated repeatedly that it may be used to maintain young rats in good condition provided the deficiency of water-soluble B is not allowed to extend over too long a period.

#### EXPERIMENTAL.

*Experiment 1.*—Test of *B. paratyphosus* B for the water-soluble vitamine. In this experiment starch bearing the bacteria was used to replace an equivalent amount in the basal ration. The weight of wet bacteria added in this way to the diet was about 600 mg. per 100 gm. of ration. In Chart 1 is graphically represented the result of this test on two animals. It will be seen that the continued loss of weight of the animals was not prevented.

*Experiment 2.*—Test of *B. coli* for the water-soluble vitamine. The starch bearing the bacteria was added as in the above experiment and the weight of organisms was in this case approximately the same. Chart 2 shows the effect of this treatment on the weight curves of two of the rats. Again the loss of weight was not prevented.

*Experiment 3.*—Test of *B. subtilis* for vitamine. In this case the organisms were grown on nutrient agar, scraped off, weighed, and added to the basal ration. 20 gm. of bacteria were added to each 100 gm. of diet. It was realized that the nutrient agar itself contained the water-soluble vitamine and it was at first thought that this might be a source of error in the interpretation of the experiment. In Chart 3 are the weight curves of three rats limited to this diet. It will be noted that the loss of weight during the period before and after the addition of the bacteria to the diet is continuous.



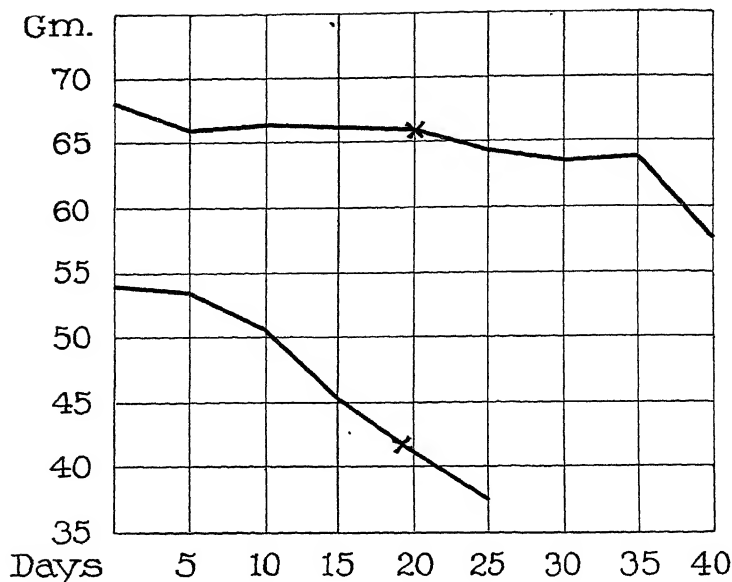


CHART 1. Up to the point marked (x) the rats were limited to a diet devoid of water-soluble B. At this point the bacteria were added to the diet. It will be seen that the loss of weight continues.

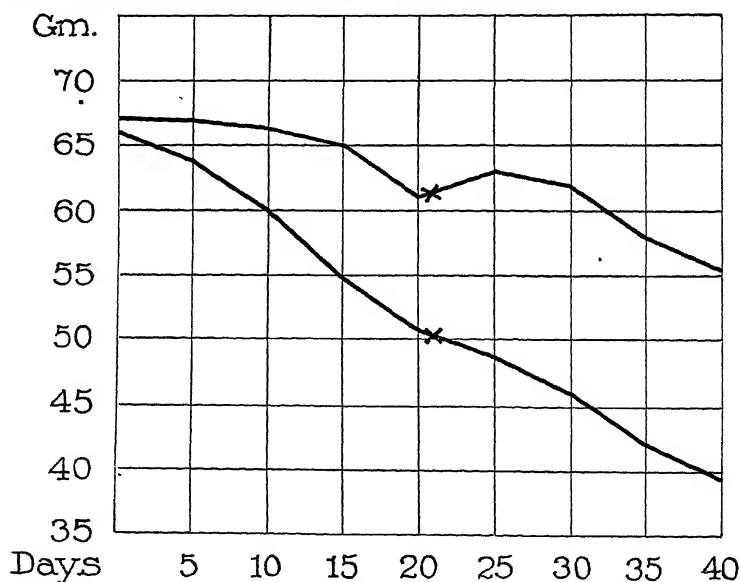


CHART 2. The preliminary diet was the same as in Experiment 1 but at the point indicated (x) *B. coli* were added to the ration. The loss of weight was not stopped.

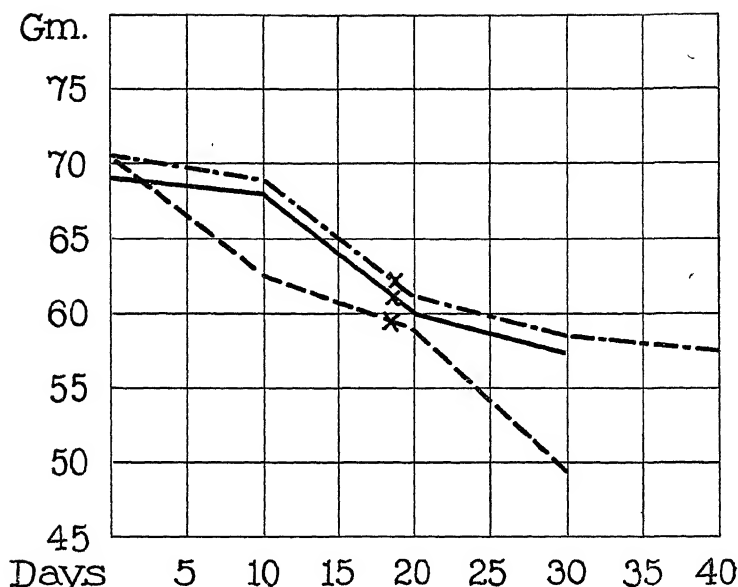


CHART 3. At the point marked (x) 20 gm. of *B. subtilis* were added to each 100 gm. of ration but the animals continued to lose weight.

#### DISCUSSION.

As already pointed out certain observers have apparently demonstrated the presence of a growth-promoting principle in bacteria. The standards by which they determined the presence or absence of this substance are not comparable however. So far as the author is aware the only way of measuring vitamine B that is not open to objection is by feeding the substance that is to be tested to rats that are being maintained on a diet that is devoid of this factor.

The experimental data presented above indicate that so far as *Bacillus paratyphosus* B, *Bacillus coli*, and *Bacillus subtilis*, are concerned there is no production of vitamine by these organisms. The objection may be raised that the quantity of bacteria added to the diet was not sufficient to affect the growth of the rats but it should be borne in mind that excessively small amounts of substances containing vitamine have been shown to produce a marked effect.

Regarding Thjötta's seeming demonstration of the production of a growth-promoting principle by mucoid bacilli and *Bacillus proteus* a further report will be made in a subsequent paper.

#### CONCLUSION.

*Bacillus paratyphosus* B, *Bacillus coli*, and *Bacillus subtilis* do not produce the growth-promoting principle known as water-soluble B vitamine.

The author desires to acknowledge the receipt of helpful suggestions from Professor F. P. Gorham.

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# THE CHARACTERISTICS OF CERTAIN PENTOSE-DESTROYING BACTERIA, ESPECIALLY AS CONCERNS THEIR ACTION ON ARABINOSE AND XYLOSE.\*

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## PLATES 1 AND 2.

(Received for publication, August 2, 1921.)

Pentoses and pentose-yielding substances are found wherever the conditions are suitable for the growth of higher plants. A study of the changes these substances undergo indicates that there are present everywhere microorganisms which are able to bring about decomposition of the pentoses and related compounds. The almost universal distribution of the five-carbon compounds and the large quantities of plant products stored up in this form indicate the importance of studying the factors concerned in their decomposition.

The great family of organisms termed the lactic acid bacteria includes many forms which possess the power of splitting vigorously the pentose sugars. Perhaps the first recognition of the rôle of the lactic acid bacteria in the fermentation of pentoses was in 1894 when Kayser (1) isolated from sauerkraut an organism which fermented arabinose and xylose with the production of lactic acid. Since this time many reports of the fermentation of pentoses have appeared, Grimbert (2), Bertrand (3), and Bendix (4).

Gayon and Dubourg (5) studied the carbohydrate metabolism of the "mannit-forming bacteria" in considerable detail. Dubourg (6) reported additional studies of these organisms. Müller-Thurgau and Osterwalder (7, 8) greatly expanded the studies of Gayon and Dubourg, isolating and describing many new strains of pentose-fermenting bacteria of the general group called "mannit-forming bacteria." They also investigated the nature of the

\* This work was in part supported by a grant from the special research fund of the University of Wisconsin.

products obtained from this fermentation. Henneberg (9) described many types of pentose-fermenting bacteria of the lactic acid group. In general, the properties of the organisms are not described in sufficient detail to follow in the identification of unknown forms of lactic acid bacteria.

Orla-Jensen (10) in an exhaustive treatise on the lactic acid bacteria gives the results of a careful study of the protein metabolism, and the fermentation characters of 330 strains of lactic acid bacteria isolated from sour cabbage, beets, sliced potatoes, mash, and dough, and also from the excrement of cows, calves, and human beings.

We have shown in preceding papers (11, 12, 13) that a certain group of the lactic acid bacteria break down the pentose sugars with a high acid production, and the hexose sugars with a low acid and a high alcohol production. Since these first reports concerning the acid fermentation of xylose, our studies have been extended until at present we have results to show that there are other types of pentose-fermenting lactic acid bacteria commonly present in silage, sauerkraut, and related substances.

The present paper deals with the fermentative ability and general characteristics of a few members of the lactic acid family which destroy pentoses. It was the hope that such an investigation would give an insight into the physiology, the distribution, and the importance of these organisms in nature.

The cultures were obtained from various samples of corn silage and sauerkraut taken at different stages of their fermentation, usually between the 10th and 21st day. The structure of the colonies on the plates was studied and different types picked off into litmus milk and into measured amounts of 1 per cent xylose-yeast water and 1 per cent glucose-yeast water. From those showing acid production in xylose-yeast water, or a curd in litmus milk after 10 days, 12 were selected and replated. The selection of 12 cultures from the large number was based chiefly on the amount of acid formed from xylose, on the change noted in litmus milk, and on the source of the culture. It was planned to have representative types of the pentose fermenters which showed decided differences in degree of acid formation.

These 12 cultures fall into two groups; the organisms of Group I are readily distinguishable from those of Group II by their action

on milk and fructose. All of the strains of Group I coagulate milk slowly and do not form mannitol from fructose. Those of Group II form mannitol from fructose and do not coagulate milk. On the basis of fermentation reactions the bacteria of Group I may be divided into at least three strains, which differ with respect to the fermentation of certain carbohydrates. It is believed that the groups herein described are fairly representative of the rod forms of lactic acid bacteria that take part in the fermentation of pentose sugars.

### *Morphology of the Cultures.*

Although morphology is of little value in subdividing any group of organisms, it is possible by such a study to divide the lactic acid bacteria into coccus forms and short and long rods. All microscopical examinations were made from 24 hour glucose or xylose-yeast water agar slants or liquid-yeast water cultures incubated at 28°C.

Chinese ink preparations are especially useful in the study of morphology. It was noted that the organisms of the different groups and strains varied but slightly in size and shape. They are non-motile, usually blunt ended rods which occur singly or in twos, although long filaments or chains are sometimes noted, especially in liquid media. The different strains vary somewhat in size, especially in width of cells, from 0.5 to 0.8  $\mu$  wide and 1.25 to 3.00  $\mu$  long; Cultures 102, 31, and 32 are smaller, about 0.5 to 0.6  $\mu$  wide by 1.2 to 2.00  $\mu$  long. Even a single culture exhibits a wide variation in the size of the cells, hence morphology is of little varietal significance. Spores are not formed. The organisms stain easily with the ordinary aniline dyes and are Gram-positive. Photomicrographs of representative fields from microscopic preparations of the various groups are shown in Plates 1 and 2, Fig. 1, 2, 3, 4, and 5.

In accordance with the results of Beijerinck (14), these various groups of lactic acid bacteria were found catalase-negative; *i.e.*, without the ability to break down hydrogen peroxide.

The cultural characteristics of these organisms did not bring out any well defined differences. Briefly: They all grow best in a medium in which the source of nitrogen is yeast-water extract. Colonies are small and reach their maximum growth in

3 to 4 days. In stab cultures growth is moderate and uniform along the line of inoculation; on slants, scanty and beaded. Gelatin is not liquefied nor is casein digested. Growth in the acid range for both groups is stopped at a hydrogen ion concentration of about pH 3.5, while in the alkaline range for Group I it is stopped at about pH 9.0 to 9.4, and for Group II at pH 8.6 to 8.8. The optimum temperature for most of the organisms is about 30°C. and their thermal death-point is between 60 and 65°C.

The group, strain, and laboratory number, the source and the behavior in milk of these cultures are shown in Table I. For the sake of comparison, cultures of *Bacillus lactis acidii* and *Bacillus bulgaricus* are included. The value of milk in dividing these groups of bacteria is well illustrated in the figures of this table. Perhaps no other single physiological test so clearly defines the groups of lactic acid bacteria that ferment pentose sugars. As compared with *Bacillus lactis acidii* or *Bacillus bulgaricus*, none of these organisms forms large amounts of acid in milk; about 0.5 per cent of lactic acid is the maximum production after 2 weeks at 28°C. The time of curdling varies with the different cultures and with the temperature. At 38°C. curdling was more rapid than at 28°C. Group I, Cultures 29, 102-1, 124-1, 102, 31, and 32 require from 8 to 18 days, while Group II, Cultures 52, 52-7, 14, 57, and 118-8 fail to curdle milk. Unlike *Bacillus lactis acidii* the organisms of Group I did not reduce litmus until after curdling.

None of the lactobacilli of the pentose-fermenting group produced a firm curd so characteristic of the lactics commonly found in milk. On the contrary, the curd is soft and flocculent and it sinks leaving a  $\frac{3}{4}$  inch layer of whey on top.

*Sources of Nitrogen.*—All attempts to grow these various strains of lactic acid bacteria in peptone-phosphate medium (0.5 per cent each of dipotassium phosphate, peptone, and xylose), and in meat infusions resulted in a slow and scanty growth. Yeast water extract is far more suitable for growth and acid production than any other medium tested and for this reason it was made use of in all of these studies. To insure a medium low in fermentable carbohydrates and also low in organic acids, only fresh yeast was used and each batch of medium was carefully analyzed. A representative analysis was as follows: 0.15 cc. of N volatile acid, 0.75 cc. of N non-volatile acid, and 0.0508 gm. of nitrogen in 100 cc. of yeast water.

TABLE I.  
The Characteristics of the *Penicillium* Fermenters in Milk.

Group.	Strain.	Culture No.	Source of cultures.	Milk at 28°C.				
				0.1 N acid.	Time of curdling.	Kind of curd.	Effect on litmus.	
I	A	29 102-1	Sauerkraut. "	cc.	days	Soft, whey on top. " " "	Reduced after curdling. " "	
				46.8 55.6	14 9			
I	B	124-1 124-2	Sauerkraut. "	46.0 24.0	9 18	Soft, whey on top. " " "	Reduced after curdling. " "	
I	C	102 31 32	Sauerkraut. Corn silage. "	18.8 40.0 37.2	18 9 9	Soft, whey on top. " " " " " "	Reduced after curdling. " " white granules at top. Reduced after curdling and white granules at top.	
II		14 118-8 57 52 52-7	Corn silage. Sheep manure. Corn silage. Sauerkraut. "	No growth in milk.				
				"	"			
				"	"			
				"	"			
				"	"			
		<i>B. lactis acid.</i> <i>B. vulgaricus.</i>		82.8 178.8	2 2	Firm, without whey on top. Firm, without whey on top.	Reduced before curdling. " "	



## PART I.

*The Fermentation of Carbohydrates and Related Substances.*

The question of the constancy of the acid fermentation of sugars by bacteria has received much study. The value of this test, as well as its limitations, has been the object of so many careful investigations that no attempt is made in this report to review the literature. The results of various investigations (15-19) indicate that the power of an organism to form acid from carbohydrates or related substances is a characteristic not easily lost or acquired.

Although in this work no extensive study has been made of variations in fermentation reactions, it has been noted that cultures of *Lactobacillus pentoaceticus* which have been carried on glucose yeast-water agar for more than 2 years have not shown any well defined change of their fermentation reactions. The acid fermentation of sugars is without doubt the best possible means of differentiating members of the lactic acid group, provided a uniform and reliable method of determining fermenting power is used.

In a study of the acid production of these bacteria, 2 per cent solutions in yeast water of the carbohydrate or related substance to be fermented were employed. The 10 day cultures were titrated for total acidity, at which time a portion of the culture was removed for hydrogen ion determination and another portion for sugar analysis. The colorimetric method for the measurement of hydrogen ion concentration and the Shaffer and Hartmann (20) method of sugar analysis were used. To overcome as much as possible the decomposition of the sugar by sterilization, the more unstable compounds, arabinose and xylose, were sterilized in water solutions and added to the yeast water by means of sterilized pipettes. The xylose was prepared from corn cobs according to the method of Monroe (21) and recrystallized from alcohol until the correct specific rotation and freedom from heavy metals had been obtained. The other sugars, alcohols, etc., were Difco or Pfanstiehl preparations and were assumed to be true to label.

In these yeast water cultures, determinations of total acidity proved far more valuable as a measure of the degree of fermenta-

tion than determinations of the hydrogen ion concentration. Within a certain range, a change in the concentration of hydrogen ions is proportional to the acid formed, but does not give a true picture of the degree of utilization of the carbon compound. This criticism may also be directed towards the titration of total acidity but to a much less degree. These two acid determinations plus the sugar analysis give a fairly accurate evaluation of the nature of the fermentation. Support for these statements will be found in the tables that follow.

*The Fermentation of Arabinose, Xylose, and Rhamnose.*

In Table II are assembled the analyses for acid production and sugar consumption from the breaking down of arabinose, xylose, and in the case of rhamnose, acid production alone. Where the acidity is below 0.5 to 0.6 per cent of normal acid it was assumed that there was no fermentation. A small amount of acid may be due to the action of the bacteria on the substance present in yeast water and to a slight decomposition of the carbohydrate during sterilization. In every case the figures given in this and in other tables are the result of subtracting this acid from that in the fermented culture.

All of the bacteria described in this paper ferment arabinose with the production of considerable amounts of acid. The various strains show a well defined difference in their ability to form acid; 10 days after inoculation cultures of the organisms of Strain A contain about 5.0 per cent, Strain C about 8.9 per cent, and Strain B about 11.3 per cent of normal acid, Table II. The high acid production which is characteristic of Strain B is also noted in the case of all of the organisms of Group II. A point of special interest shown in the results presented in this table is the close agreement between sugar fermented and acid formed. Perhaps in no other table is this correlation so evident. These results indicate that the chief products of the fermentation are acids rather than neutral or highly volatile bodies. *Bacillus lactis acidi* did not ferment arabinose, xylose, or rhamnose. On the basis of total acid formed from xylose, the bacteria of Group I may be arranged in three divisions: Strain A and B, low acid, and Strain C, no acid, or only a trace. All organisms of Group II are high acid formers.

TABLE II.  
*The Fermentation of Pentoses.*

Calculated for 100 cc. of culture.											
Group.	Strain.	Culture No.	Arabinose.			Xylose.			Rhamnose.		
			0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH	Sugar fermented.	0.1 N acid.	pH	
			cc.		gm.	cc.		gm.	cc.		
I	A	29 102-1	53.1	4.0	0.410	47.5	3.8	0.388	37.0	5.6	
			46.5	3.8	0.257	39.9	3.8	0.187	35.6	4.0	
I	B	124-1 124-2	114.0	3.2	0.950	58.4	3.8	0.375	21.6	4.2	
			113.0	3.2	0.950	58.9	3.8	0.355	25.4	4.0	
I	C	102	86.0	3.6	0.554	6.1	4.6	0.014	7.5	5.4	
		31	91.5	3.4	0.530	7.0	4.8	0.018	34.8	3.8	
		32	89.0	3.4	0.596	7.5	4.8	0.045	36.4	3.8	
II		14	122.4	3.2	0.950	105.3	3.2	0.874	6.5	5.6	
		118-8	124.3	3.2	0.950	94.7	3.2	0.874	5.4	6.0	
		57	118.5	3.4	0.950	102.0	3.4	0.874	6.8	5.6	
		52	103.8	3.4	0.950	104.2	3.4	0.874	7.2	5.4	
		52-7	111.0	3.2	0.950	104.1	3.2	0.874	1.4	6.4	
<i>B. lactis acidi.</i>			5.7	4.4		5.9	4.8		7.8	5.4	

Cultures 29, 102-1, 124-1, and 124-2 of Group I formed from xylose only about half as much acid as the cultures of Group II and likewise consumed much less sugar. The fermentation of xylose will be discussed more fully in a later table. Repeated tests show that Cultures 102, 31, and 32 do not ferment xylose, or if so, only to a slight degree.

Rhamnose is fermented by all of the cultures of Group I but not by any of the cultures of Group II. As compared with the other sugars the amount of acid produced from rhamnose is small.

The significant fact brought out in the figures of this table is the marked difference in fermentation of the two sugars, arabinose and xylose. In 10 days, Cultures 124-1 and 124-2 destroy 1 per cent of arabinose and form more than 11 per cent of normal acid, while on xylose less than half of this amount of sugar is consumed and 5.8 per cent of normal acid formed. Strain C, represented by Cultures 102, 31, and 32, readily attacks arabinose but not xylose.

*Progressive Fermentation of Xylose.*—The high acid from arabinose and the low acid from xylose formed by Cultures 124-1 and 124-2 cannot be explained on the ground that these organisms are sensitive to an acid reaction, for with arabinose the total acid and the true acidity are far greater than with xylose. It is possible that this strain attacks xylose much more slowly than arabinose. To test this point an experiment was made in which the formation of acid from xylose was measured at different times. Titrations after 3, 8, 10, and 30 days were made. No decided increase in acid formation was noted in the cultures kept for 30 days. Apparently the total acid after 10 days is not greatly increased by longer incubation.

It was noted that the rate of acid production is rapid, within 3 days approximately one-half of the total acid formed from xylose is obtained.

#### *The Fermentation of Glucose, Galactose, Mannose, and Fructose.*

The complete results of the fermentation of these sugars are given in Table III. The chief point of interest in the table is the high acid production by the bacteria of Group I and the low acid production by the bacteria of Group II. While the total acid

total acid of any of the organisms included in Group I. Cultures 31 and 32 break down sucrose with a high acidity, about 10.7 per cent of normal acid, maltose with a much lower total acidity about 8.7 per cent, and lactose with a high total acidity of about 10.8 per cent.

In the fermentation of these three disaccharides, the organisms of Group II behaved very differently from those of Group I. In

TABLE IV.  
*Fermentation of Disaccharides.*

Culture No.	Calculated for 100 cc. of culture.								
	Sucrose.			Maltose.			Lactose.		
	0.1 N acid.	pH	Sugar fer- mented.	0.1 N acid.	pH	Sugar fer- mented.	0.1 N acid.	pH	Sugar fer- mented.
	cc.		gm.	cc.		gm.	cc.		gm.
29	94.0	3.4		95.0	3.4	0.955	102.7	3.4	0.930
102-1	101.0	3.4		107.5	3.2		92.0	3.4	
124-1	118.0	3.4		103.8	3.4		87.5	3.4	1.060
124-2	120.0	3.4	0.946	107.3	3.4	1.070			
102	86.0	3.4	0.583	78.5	3.4	0.960	71.0	3.6	0.670
31	108.0	3.4		87.0	3.6		111.0	3.3	
32	106.0	3.4		87.3	3.4		106.5	3.4	
14	54.5	3.6	0.500	61.5	3.6	1.150	23.0	4.4	0.380
118-8	42.2	3.6	0.205	61.0	3.6	1.118	8.0	5.6	
57	41.1	3.6		56.3	3.6		6.0	5.8	
52	19.9	4.6		65.0	3.6	1.070	6.0	5.6	
52-7	10.1	5.6		60.0	3.6		7.0	5.6	
<i>B. lactis acid.</i>				61.6	3.4		102.0	3.4	

general these mannitol-forming bacteria produced about 5.6 to 6.5 per cent of normal acid from maltose, a somewhat lower percentage of acid from sucrose, and only a trace of acid from lactose. Culture 14 offers an exception, it attacks lactose. Although not all the cultures of Group II break down sucrose and maltose with the same final yield of acid, the differences are not great enough to be of use in dividing this group. Apparently Culture 14 possesses a slightly different fermentative ability from any of the

other members of this group. If acid production of these various groups of bacteria be compared with that of *Bacillus lactis acidi* it will be seen that there is a decided difference; the latter attacks maltose slowly, and lactose readily.

From the sugar determinations, it appears that in certain cases the sugar consumed is not proportional to the acid formed. Perhaps neutral substances are formed during the fermentation. For an example, see the figures obtained from the fermentation of maltose by the bacteria of Group II.

*Progressive Fermentation of Lactose.*—The rate of the fermentation of lactose was measured after 4, 7, and 10 days. It was found that the fermentation proceeds so rapidly that within 4 days after inoculation the greater part of the acid is formed. From the 4th to the 10th day the acid content increases but slowly. If calculated as lactic acid the total sugar fermented is approximately equal to the acid formed. One point of special interest noted in the results of this fermentation is the variation in acid production of the organisms of Strain C. It was noted that the total acid formed by Culture 102 from lactose is much less than that formed by Cultures 31 and 32.

*The Fermentation of Melezitose, Raffinose,  $\alpha$ -Methyl Glucoside, Salicin, and Esculin.*

The results of the fermentation of the trisaccharides, melezitose and raffinose, and the glucosides,  $\alpha$ -methyl glucoside, salicin, and esculin, are given in Table V. The trisaccharide, melezitose, is not easily decomposed. Because of the decided difference in the availability to these organisms as measured by the production of acid, melezitose is an important sugar in the differentiation of these bacteria. The organisms of Strain C attack this trisaccharide vigorously, producing about 10 to 11 per cent of normal acid from a 1 per cent solution of the sugar. It is significant that with the exception of a very small acid production by the organisms of Strain B none of the other bacteria attacked melezitose.

Raffinose is far more available for the lactic acid bacteria than melezitose. Except in the case of the organisms of Group II and *Bacillus lactis acidi*, raffinose is decomposed with the production of large amounts of acid. It does not furnish any characteristic

fermentation reactions for the pentose-destroying bacteria of Strains A, B, and C.

$\alpha$ -Methyl glucoside is readily fermented by the majority of the organisms of Groups I and II. The various strains of Group I exhibit well defined differences in their power of acid production from this glucoside; Strain B forms large amounts of acid, Strain A approximately half as much acid as Strain B, and Strain C does

TABLE V.  
*Fermentation of Trisaccharides and Glucosides.*

Culture No.	Calculated for 100 cc. of culture.								
	Melezitose.		Raffinose.		$\alpha$ -Methyl glucoside.	Salicin.		Esculin.	
	0.1 N acid	pH	0.1 N acid.	pH	0.1 N acid.	0.1 N acid.	pH	0.1 N acid.	pH
	cc.		cc.		cc	cc.		cc.	
29	9.4	5.6	91.6	3.4	21.8	81.6	3.4	36.3	4.4
102-1	7.6	5.2	83.6	3.5	23.1	91.2	3.4	36.3	4.2
124-1	10.0	5.2	82.8	3.6	48.0	80.6	3.6	38.2	4.2
124-2	11.6	5.2	83.0	3.6	60.5	80.0	3.6	38.2	4.0
102	106.4	3.6	83.6	3.6	7.6	90.6	3.6	34.8	4.0
31	110.4	3.4	81.6	3.6	7.6	56.2	3.6	36.4	4.0
32	112.6	3.2	83.8	3.4	7.1	78.0	3.6	33.5	4.4
14	6.8	5.6	2.2	6.0	43.8	12.8	5.0		7.2
118-8	7.6	5.6	5.0	5.8	47.0	5.0	6.2		7.2
57	6.4	5.8	5.0	5.8	41.8	4.0	6.2		7.2
52	8.0	5.4	5.2	5.6	45.2	3.2	5.6		7.2
52-7	5.6	6.2	6.4	5.6	46.2	2.4	6.4		7.2
<i>B. lactis acidi.</i>	9.6	5.1	6.2	5.6	16.7	21.6	4.2		7.2

not attack  $\alpha$ -methyl glucoside. Because of the decided difference in the fermentation of  $\alpha$ -methyl glucoside by the lactic acid bacteria isolated from fermenting plant tissue, it is important in subdividing the various strains of these organisms. It is not attacked by the bacteria of Strain C. All the organisms of Group II form acid from  $\alpha$ -methyl glucoside.

The two glucosides, salicin and esculin, are decomposed by the organisms of Strains A, B, and C, but are resistant to the organ-

isms of Group II. An exception to this statement is seen in the case of Culture 14 where a slight fermentation is noted. In every case the total acid formed from esculin is far less than that from salicin.

*Fermentation of Mannitol, Glycerol, and Dulcitol.*

The polyhydric alcohols, mannitol, glycerol, and dulcitol furnish sources of carbon for the separation of the lactic acid bac-

TABLE VI.  
*Fermentation of Polyhydric Alcohols.*

Culture No.	Calculated for 100 cc. of culture.					
	Mannitol.		Glycerol		Dulcitol.	
	0.1 N acid.	pH	0.1 N acid.	pH	0.1 N acid.	pH
	cc.		cc.		cc.	
29	42.0	3.5	36.0	3.8	6.2	5.4
102-1	56.0	3.5	33.2	3.9	6.0	5.4
124-1	49.0	3.6	25.6	4.0	39.8	3.8
124-2	58.0	3.7	27.0	4.0	36.4	3.8
102	46.0	3.6	17.6	4.4	6.0	5.4
31	57.0	3.6	14.6	4.0	4.6	5.8
32	55.0	3.6	13.8	4.2	5.6	5.6
14	20.0	4.4	8.6	5.8	4.0	6.6
118-8	15.2	4.3	5.4	6.0	3.6	6.2
57	15.6	4.4	5.0	6.2	3.4	6.6
52	16.2	4.2	4.8	6.0	3.8	6.6
52-7	19.0	4.2	3.8	6.0	3.6	6.4
<i>B. lactis acidi.</i>	46.0	3.8			7.4	5.6

teria into groups, as shown in Table VI. Of the three alcohols dulcitol is by far the most important for a study of fermentation reactions. It is not attacked by any of the lactic acid organisms used in this study except Cultures 124-1 and 124-2. In this respect our results agree with those of Orla-Jensen (10) who says that lactic acid bacteria which ferment dulcitol are extremely rare. According to Winslow and his associates (23) dulcitol occupies a unique position in the fermentation test in that it does not corre-



late with the other carbohydrates. From our results it appears that the fermentation of dulcitol is highly specific and may be used to separate closely related strains of lactic acid bacteria. Mannitol is fermented by all of the organisms of both groups, although much more slowly by those of Group II. Glycerol is fermented even more slowly than mannitol and very slightly by the organisms of Group II.

In addition to the substances already described fermentation tests were carried out with starch, dextrin, and inulin, but no appreciable acid production was noted.

#### *Distinctive Fermentation Characteristics.*

The fermentation of certain carbohydrates and related compounds furnishes a means of separating into well defined groups the lactic acid bacteria that ferment the pentose sugars. Arabinose, xylose,  $\alpha$ -methyl glucoside, melezitose, and dulcitol have been found especially useful in the separation of the different strains of Group I. 1 per cent xylose-yeast water is easily the most valuable medium in the separation of these lactic organisms into different groups. According to the amounts of acid formed these organisms naturally fall into three, and possibly four, divisions. The separation of Strains A and B, is based solely on the variation in the amount of acid produced, approximately 4.4 per cent of normal acid for Strain A, and 5.8 per cent for Strain B. It is only fair to say that the fermentation of xylose does not furnish a clear-cut separation of the organisms of these two groups. The production of acid from dulcitol is an easily distinguishable characteristic of the organisms of Strain B, while those of Strain A will not attack this polyhydric alcohol. The bacteria of Strain C are readily separated from the other organisms of both groups by the fermentation of melezitose.

The principal results of the fermentation tests are grouped in Table VII. The figures of this table bring out clearly the most interesting facts obtained in this preliminary study of the pentose-fermenting lactic acid bacteria. Here the fermentation of the test substance is indicated by numbers. The data are arranged to bring out the degree of fermentation. For example, in the case of Culture 29 the figure 3 for arabinose and xylose indi-

cates a fair acid production while 6 for hexoses and disaccharides indicates a strong fermentation. The figures of this table show the approximate amount of acid formed by the several organisms from the breaking down of the various carbohydrates and related compounds.

TABLE VII.  
*General Fermentation Characters of the Pentose Bacteria.*

Carbon compound.	Group I.						Group II.					
	A		B		C							
	29	102-1	124-1	124-2	102	31	32	14	118-8	57	52	52-7
1. Arabinose.....	3*	3*	6*	6*	5*	6*	5*	6	6	6	6	6
2. Xylose.....	3*	2*	3*	3*	0*	0*	0*	6	6	6	6	6
3. Rhamnose.....	2	2	1	1	0	2	2	0	0	0	0	0
4. Glucose.....	6	6	6	6	6	6	6	5	4	4	4	5
5. Galactose.....	6	6	6	6	5	6	6	3	3	3	3	3
6. Mannose.....	6	6	6	6	6	6	6	2	1	1	1	1
7. Fructose.....	6	6	6	6	6	6	6	4	4	4	4	4
8. Sucrose.....	6	6	6	6	5	6	6	3	2	2	1	0
9. Maltose.....	6	6	6	6	5	5	5	4	4	3	4	4
10. Lactose.....	6	6	5	—	4	6	6	1	0	0	0	0
11. Melezitose.....	0*	0*	0*	0*	6*	6*	6*	0	0	0	0	0
12. Raffinose.....	6	5	5	5	5	5	5	0	0	0	0	0
13. $\alpha$ -Methyl glucoside.....	1*	1*	3*	4*	0*	0*	0*	2	3	2	2	3
14. Salicin.....	5	5	5	5	6	3	5	0	0	0	0	0
15. Esculin.....	2	2	2	2	2	2	2	0	0	0	0	0
16. Mannitol.....	2	3	3	3	3	3	3	1	1	1	1	1
17. Glycerol.....	2	2	1	1	0	0	0	0	0	0	0	0
18. Dulcitol.....	0*	0*	2*	2*	0*	0*	0*	0	0	0	0	0

\* These figures represent the fermentation tests especially useful in separating the different strains of Group I.

The arbitrary standards adopted were as follows:

0 equals 0—15 cc. of 0.1 N acid.

1 “ 15—30 “ “0.1 “ “

2 “ 30—45 “ “0.1 “ “

3 “ 45—60 “ “0.1 “ “

4 “ 60—75 “ “0.1 “ “

5 “ 75—90 “ “0.1 “ “

6 “ 90 or above cc. of 0.1 N acid.

Among the compounds most valuable for differentiation are lactose, fructose, melezitose, the pentoses, and the higher alcohol, dulcitol. This separation is not dependent upon acid formation alone but also on the production of neutral bodies, for example, mannitol from fructose. It is realized that the grouping adopted in this paper may bring together bacteria related in only one character but not in other characters; however, it is the best means at hand to separate the great complex of lactic acid bacteria.

## PART II.

### *Quantitative Determination and Identification of the Products Formed from Arabinose and Xylose.*

Arabinose and xylose are easily destroyed when sterilized in a slightly alkaline solution, and undergo a small amount of decomposition even when sterilized in yeast water. To avoid this change an 8 per cent water solution of each pentose was sterilized and then by means of a sterilized pipette added to the yeast water until the concentration was about 2 per cent. The exact strength was determined by analysis, which usually gave from 1.91 to 2.04 per cent of the pentose. The fermentation flask was equipped with a carbon dioxide absorption bottle similar to that described in a previous paper (12). Sterilized brom-cresol purple was added to the fermenting solution at the time of inoculation to indicate the formation of acids. Whenever a strong acid reaction was apparent, sterilized N sodium hydroxide was added until the solutions were approximately neutral. In this way, it was possible to measure the rate of fermentation and also to determine when fermentation had ceased. Occasionally fresh additions of brom-cresol purple were necessary as the indicator is partly destroyed in the fermentation process. When acid formation ceased, usually after 10 to 14 days, the cultures were analyzed for carbon dioxide, unfermented sugar, volatile and non-volatile acids, and alcohol.

*Methods of Analysis.*—Carbon dioxide, volatile and non-volatile acids, and alcohol were determined as described in previous publications (11, 12). The distillate was analyzed by the method of von Fürth and Charnass (24) for lactic acid carried over with

the volatile acid, but in no case were more than a few milligrams found. A correction corresponding to the quantity found has been applied to the values for acetic and lactic acids.

Sugars were determined by the titration method of Shaffer and Hartmann (20). This is a rapid volumetric method that gives practically the same accuracy as the longer gravimetric methods. The method was tested with purified xylose and arabinose and found to give quantitative recovery of the sugars both from water and from culture solutions.

Analyses were made of the uninoculated medium for volatile and non-volatile acids, blanks were run on the reagents, and the values thus obtained were subtracted from the corresponding determinations of the fermented cultures.

In the regular procedure 400 cc. of an approximately 2 per cent sugar solution were fermented. Due to the addition of *N* NaOH to neutralize the acids formed, the volume at the end of the fermentation was in the neighborhood of 475 cc. Of this, 200 cc. were used for the determination of volatile and non-volatile acids, 100 cc. for alcohol, 50 cc. for sugar, 5 to 10 cc. for carbon dioxide, and the remainder kept in the ice box as a reserve in case a determination should be lost.

The fermentation of the pentoses was rapid and usually complete in about 14 days. Of the two, arabinose was more rapidly fermented than xylose. Very little sugar remained unfermented, rarely more than 0.1 gm. and sometimes as little as 0.05 gm. to 100 cc. of culture. Of the fermented sugar about 95 per cent is accounted for by the products. The volatile and non-volatile acids, which later will be shown to be acetic and lactic acid respectively, comprise 98 per cent or more of the total products. No measurable quantity of alcohol was found and the carbon dioxide was never more than 0.046 gm. for 100 cc. of culture. The acetic acid and lactic acid are produced at approximately the ratio of 1 molecule of acetic for 1 molecule of lactic. The ratio of their molecular weights is 1:1.50 and the ratios found vary from 1:1.34 to 1:1.52. Somewhat lower ratios were obtained from the mannitol-forming group, the bacteria of which have the power of slowly fermenting lactic acid to acetic acid and carbon dioxide. When all, or nearly all, of the sugar has been fermented, it is probable that the bacteria attack the lactic acid formed and

so increase the proportion of acetic acid at the expense of the lactic acid. The absence of any appreciable amount of arabinose or xylose (less than 0.05 gm. to 100 cc. of culture) and a rather high production of carbon dioxide lends support to this view. The data are given in Table VIII.

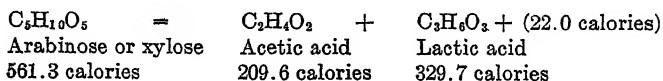
TABLE VIII.

*Total Fermentation Products from Arabinose and Xylose.*

Culture No.	Sugar.	Calculated for 100 cc. of culture.					
		Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ratio of acetic to lactic.	Carbon dioxide.	Sugar accounted for by products.
		gm.	gm.	gm.		gm.	per cent
29	Arabinose.	1.73	0.653	0.992	1:1.52	0.023	96
29	Xylose.	1.79	0.695	1.022	1:1.48	0.023	97
124-2	Arabinose.	1.81	0.688	1.004	1:1.46	0.017	90
124-2	Xylose.	1.73	0.644	0.936	1:1.45	0.024	93
102	Arabinose.	1.97	0.714	1.035	1:1.45	0.023	90
31	"	1.95	0.660	0.968	1:1.47	0.031	85
14	"	1.97	0.705	1.015	1:1.44	0.047	90
14	Xylose.	1.94	0.771	1.043	1:1.36	0.030	95
118-8*	"	1.41	0.545	0.732	1:1.34		91
52-7	"	1.94	0.780	1.082	1:1.39	0.021	97

\* From *J. Biol. Chem.*, 1919, xxxix, 368.

Aside from this somewhat lower ratio between acetic and lactic acids for the mannitol-forming group, no essential difference manifests itself in the splitting of the two pentoses by the different bacteria. The sugars are almost completely fermented in all cases; carbon dioxide is produced only in minute quantities, and acetic and lactic acids constitute almost the entire amount of measurable products. On the basis of these data the fermentation equation may be represented as:



A certain quantity of the sugar is incorporated in the cells of the bacteria and a small quantity is consumed for their development, but the foregoing equation is as nearly quantitative as can be expected of a biological process.

### *Identification of Products.*

*Volatile Acid.*—The volatile acid from the various fermentations was subjected to a Duclaux distillation and the distilling constant calculated from the titration data. The constants obtained are given in Table IX. For comparison the distilling constant obtained with our apparatus for acetic acid made from recrystallized barium acetate and Duclaux's original constant for acetic acid are also given in this table. The results indicate that the volatile acid is practically pure acetic acid in all cases, although the constants are slightly higher than for acetic acid. The difference is within the range of experimental error. Slight variation in the constants is to be expected with different pieces of apparatus, and different methods of heating. Additional evidence for the absence of a higher fatty acid such as propionic acid was obtained by fractionating a volatile acid distillate, Arabinose Culture 14, containing 200 cc. of 0.1 N acid. The barium salt was dissolved in 100 cc. of water, 50 cc. of 0.1 N sulfuric acid were added, and the partially freed volatile acid was distilled with steam. The higher fatty acids such as propionic and butyric would, if present, be concentrated in the distillate leaving the lower acids in the distilling flask. The fractional distillate was submitted to a Duclaux analysis, but proved to have the same distilling constant as the unfractionated distillate. Since there was no change in the distilling constant, it is evident that the first distillate contained a single volatile acid and not a mixture.

As a check on the Duclaux analysis the barium content of the volatile acid was determined. The barium salt was dried to constant weight in a platinum dish at 130°C. and then ignited with an excess of sulfuric acid. The weights of barium sulfate found and the calculated quantity that should have been present if the salt was barium acetate are given in Table X. The close agreement between the found and theoretical values proves that the volatile acid was acetic and corroborates the conclusions of the Duclaux analysis.

TABLE IX.  
*Distilling Constants of the Volatile Acids Obtained by Duclaux Method.*

Culture No.	Source of acid.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
29	Arabinose.	7.5	15.4	23.7	32.4	41.4	51.0	61.2	72.1	84.9	100.0
29	Xylose.	7.5	15.5	23.8	32.5	41.6	51.1	61.4	72.6	85.1	100.0
124-2	Arabinose.	7.5	15.5	23.8	32.5	41.5	51.2	61.4	72.6	85.0	100.0
124-2	Xylose.	7.5	15.4	23.9	32.3	41.3	50.9	61.2	72.4	85.0	100.0
102	Arabinose.	7.4	15.5	23.8	32.4	41.4	50.9	61.3	72.4	85.1	100.0
31	"	7.6	15.5	24.0	32.7	41.8	51.4	61.7	72.8	85.3	100.0
14	"	7.6	15.6	24.0	32.8	41.9	51.5	61.7	72.9	85.3	100.0
14	" *	7.6	15.6	24.0	32.6	41.8	51.3	61.6	72.6	85.2	100.0
14	Xylose.	7.7	15.7	24.1	32.8	42.0	51.4	61.9	73.0	85.5	100.0
Author's constant for purified acetic acid.		7.4	15.2	23.6	32.2	41.4	51.0	61.3	72.4	85.1	100.0
Duclaux constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100.0

\* First fraction of total acid used in preceding distillation—Arabinose Culture 14.

*Non-Volatile Acid.*—The barium salts were evaporated to dryness, taken up with 10 to 15 cc. of water, filtered, and absolute alcohol was added to the filtrate, as in the Möslinger method (25), until a concentration of 90 per cent alcohol was reached. In most cases a slight precipitate of some amorphous material was formed. A similar precipitate obtained by extracting the uninoculated control showed it to be an impurity from the yeast water and not the barium salt of some non-volatile acid produced by fermentation. A portion of the alcohol solution was evaporated to dryness and dried at 130°C., and the barium content of the residue determined as in the case of the volatile acid. The data are given in Table XI and agree satisfactorily

TABLE X.  
*Composition of Barium Salts of the Volatile Acids.*

Sugar fermented.	Culture No.	Barium salt of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		gm.	gm.	gm.
Arabinose.	29	0.5680	0.5132	0.5189
Xylose.	29	0.3128	0.2812	0.2858
Arabinose.	124-2	0.5983	0.5448	0.5466
Xylose.	124-2	0.7002	0.6370	0.6397
Arabinose.	102	0.3880	0.3530	0.3545
“	31	0.6910	0.6288	0.6314
“	14	0.7560	0.6890	0.6908
Xylose.	14	0.3354	0.3026	0.3065

with those required for lactic acid. The optical form of the lactic acid was ascertained by preparing the zinc lactate and determining its water of crystallization. Inactive zinc lactate crystallizes with 3 molecules of water, or 18.17 per cent, while the active form contains only 2 molecules, or 12.9 per cent. The percentages of water found are given in Table XII and prove that all of these different bacteria produce inactive lactic acid.

In previous publications (11, 12, 13) it has been shown that the mannitol-forming bacteria of Group II as far as has been determined always form a racemic mixture of lactic acid. This is true for the fermentation of both the pentoses and hexoses and also holds for mannitol. The non-mannitol-forming bacteria, Group



I, exhibit this same characteristic toward pentoses. The kind of lactic acid produced from hexoses and hexahydric alcohols remains to be determined.

TABLE XI.

*Composition of the Barium Salts of the Non-Volatile Acids.*

Sugar fermented.	Culture No.	Barium salt of the non-volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for lactic acid.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Arabinose.	29	0.3376	0.2456	0.2498
Xylose.	29	0.2756	0.2024	0.2039
Arabinose.	124-2	0.7104	0.5268	0.5257
Xylose.	124-2	0.5156	0.3750	0.3815
Arabinose.	102	0.8040	0.5852	0.5949
"	31	0.8996	0.6578	0.6657
"	14	0.3426	0.2464	0.2535
Xylose.	14	0.3232	0.2372	0.2392

TABLE XII.

*Water of Crystallization Contained in the Zinc Lactates.*

Culture No.	Source of salt	Weight of zinc lactate used.	Water lost.		Water in Zn (C <sub>3</sub> H <sub>5</sub> O <sub>3</sub> ) <sub>2</sub> + 3H <sub>2</sub> O.
			<i>gm.</i>	<i>per cent</i>	
29	Arabinose.	0.7466	0.1342	18.0	18.17
29	"	0.3854	0.0694	18.0	18.17
29	Xylose.	0.6024	0.1072	17.6	18.17
124-2	Arabinose.	1.2256	0.2220	18.1	18.17
124-2	Xylose.	1.1942	0.2162	18.1	18.17
102	Arabinose.	2.2206	0.4042	18.2	18.17
31	"	1.7082	0.3094	18.1	18.17
14	"	1.5862	0.2870	18.1	18.17
14	Xylose.	0.6864	0.1232	18.0	18.17

## SUMMARY.

The pentose sugars, arabinose and xylose, are readily fermented by various strains of the lactic acid bacteria. These pentose-destroying bacteria are widely distributed in nature, occurring in large numbers in silage, sauerkraut, and related products. At different stages of the fermentation of corn silage and sauer-

kraut, pure cultures of these lactic acid bacteria were isolated and their general characteristics studied. It was found that these organisms are usually short, blunt ended rod forms occurring as single cells or long filaments. From a large number of cultures isolated 12 were selected for special study.

This choice of 12 cultures was based chiefly on the amount of acid formed from arabinose and xylose, on the change noted in litmus milk, and on the source of the culture. According to their behavior in milk the lactic acid bacteria which ferment pentoses may be arranged in two groups, the one group which slowly causes the milk to coagulate, the other which fails to bring about any noticeable change.

Because of the very slight variation in morphology, the separation of these organisms into groups depends upon characters other than cell structure. Measurements of the fermentative ability are undoubtedly the best means of separating the various groups and strains of the lactic acid bacteria. Among the compounds most valuable for differentiation are xylose, arabinose, fructose, lactose, melezitose, dulcitol, and  $\alpha$ -methyl glucoside. The organisms studied naturally fall into two great groups; those of Group I ferment fructose without forming mannitol, and those of Group II ferment fructose with the production of mannitol. Aside from the two main divisions these organisms may be arranged into several subdivisions or strains, depending upon differences in kind of sugars fermented and amount of acid formed.

The following group of reactions indicates the nature of these strains:

*Group I.*—Strain A ferments arabinose, xylose, and lactose, but does not ferment melezitose or dulcitol. Strain B ferments arabinose, xylose, lactose, and dulcitol, but does not ferment melezitose. Strain C ferments arabinose, lactose, and melezitose, but does not ferment xylose or dulcitol.

*Group II.*—All strains ferment arabinose and xylose, but do not ferment lactose, melezitose, or dulcitol.

No doubt some of these forms have been described previously; however, the characters reported are not in sufficient detail to insure identification.

The authors suggest the following names for these various types of lactic bacteria:

*Group I.*—Strain A—Cultures 29, 102-1, *Lactobacillus pentosus*, *n. sp.* Strain B—Cultures 124-1, 124-2, *Lactobacillus pentosus*, *n. sp.*

The authors feel that the difference between Strains A and B is not sufficient to warrant a separate name for Strain B.

Strain C—Cultures 102, 31, and 32. *Lactobacillus arabinosus*, *n. sp.*

*Group II.*—This group contains closely related organisms belonging to the *Lactobacillus pentoaceticus* type.

The fermentation of arabinose and xylose by certain of the lactic acid bacteria results in the production, mainly, of acetic acid and lactic acid. These two compounds are equivalent to about 90 per cent of the sugar destroyed and 98 per cent of the isolated products. The ratio of the two acids to one another is approximately 1 molecule of acetic to 1 molecule of lactic. The theoretical ratio of their molecular weights is 1:1.50, while the ratios found varied from 1:1.34 to 1:1.52. The mannitol-forming bacteria slowly ferment lactic acid to acetic acid and carbon dioxide. The secondary fermentation results in a deviation from the theoretical ratio in the direction of the lower values.

The only other product that could be identified was carbon dioxide. This is produced in minute quantities; from 10 to 20 mg. are formed per gm. of sugar fermented.

On the basis of the almost complete fermentation of the pentoses and the high percentage of sugar accounted for by the products, it appears that the main line of the fermentation is simple cleavage into acetic and lactic acids.

The pentose-fermenting organisms studied represent a closely related family which may be divided into groups and strains according to their fermentative ability. Although these organisms possess differential fermentation characters, the products in the breaking down of arabinose and xylose by them are identical, and in the same proportions.

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## EXPLANATION OF PLATES.

## PLATE 1.

FIG. 1. Culture 29. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.

FIG. 2. Culture 124-2. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,200.

## PLATE 2.

FIG. 3. Culture 102. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,200.

FIG. 4. Culture 31. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.

FIG. 5. Culture 14. A 24 hour culture on arabinose yeast agar. Stained with fuchsin.  $\times$  1,000.

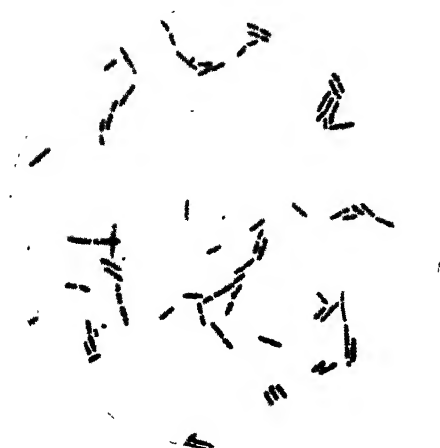


FIG. 1.



FIG. 2.

(Fred, Peterson, and Anderson: Pentose-destroying bacteria.)



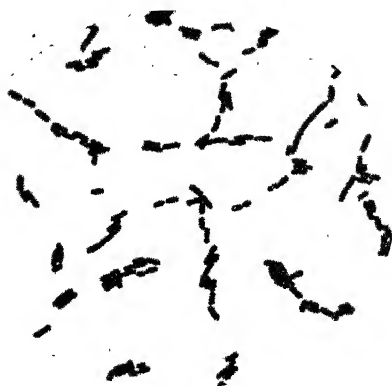


FIG. 3.



FIG. 4.

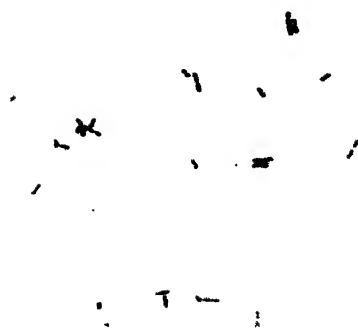


FIG. 5.

(Fred Peterson and Anderson: Pentose-destroying bacteria.)





## THE EXCRETION OF ACETONE FROM THE LUNGS.

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It is well known that under conditions which lead to the formation and accumulation of the "acetone bodies," acetone is present in the breath. It occurred to one of us that this excretion of acetone from the lungs might be the result merely of evaporation from the blood plasma into the alveolar air, conditioned by the distribution coefficient of acetone between plasma and air at the body temperature. To learn whether this surmise was correct we have determined the distribution coefficients of acetone between water and air, and blood and air outside the body at different temperatures, and with these data have compared results, and coefficients obtained therefrom, from human subjects of natural ketonemia and from dogs after the injection of acetone solutions intravenously.

Our experiments lead to the conclusion that acetone is excreted from the lungs by the simple process of diffusion and volatilization, since the ratio between the concentrations in blood and alveolar air is the same as the distribution coefficient determined *in vitro* at body temperature. A smaller number of experiments indicate that the concentration of acetone in urine is equal to and parallels its concentration in blood, and the excretion of acetone by the kidneys also thus appears to be the result of simple diffusion.

At the time of beginning our experiments about 2 years ago, we were under the impression that the question had not been taken up from the point of view mentioned, but this impression proved to be incorrect when the literature was more carefully searched.

The characteristic odor of the breath of patients showing symptoms of diabetic coma, and the fact that this odor is due at

least in part to acetone, noted by Petters (1) in 1857, is the beginning of practically all of our knowledge concerning the acetone bodies. Although most of the host of workers who have contributed to the subject have devoted their attention to the excretion of these substances by the kidneys there have been many observations on the excretion of acetone by the lungs. The first quantitative determinations of the amount thus excreted seem to have been made in 1897. In that year Nebelthau (2) recorded the excretion of 150 mg. per hour by the lungs by a subject of chronic inanition who excreted about one-tenth that amount in the urine. The same year Geelmuyden (3) found that on giving acetone by mouth or subcutaneously to rabbits and dogs it was in great part excreted by the lungs; and Schwarz (4) showed that dogs and rabbits excrete only a few per cent of injected or fed acetone in the urine while from 50 to 76 per cent appears in the breath. In the same volume in which Schwarz' paper was published, Müller (5) describes and pictures a simple apparatus with which he determined the amount of acetone excreted in the breath. The exhaled air was bubbled through ice water and the absorbed acetone titrated by the Messinger method. With normal well fed subjects he found 1.3 to 3.3 mg. of acetone per hour, with diabetics up to 20 mg. per hour, and after giving by mouth 3.8 gm. of acetone to a normal subject, 130 mg. per hour in the exhaled air. Although he records no blood analyses and no individual determinations, he concluded that the amount of acetone in the breath depends upon (1) the content of acetone (or acetoacetic acid) in the blood or tissues, and (2) the volume of respired air, the evaporation in the lung capillaries being faster the lower the acetone tension in the alveolar air and *vice versa*. It is evident that Müller had in mind that the pulmonary excretion of acetone is determined by its partial pressure in blood and alveolar air, though he offered no data to prove that such is the case. In 1910 Cushny (6), without it seems being aware of Müller's work, reported data from three experiments on cats which had received acetone intravenously, showing that the excretion in the breath was fairly constant over a 3 hour period, from 8.5 to 14 per cent being excreted within that time, depending upon the amount injected. He states that the "percentage of that injected which is excreted in a given time

thus rises with the dose." He also performed one experiment in which measured volumes of air were drawn through an acetone solution (containing chloroform as well) of known concentration and found that the rate of evaporation of acetone was similar to the rate of its excretion in exhaled air by a cat which had been injected with acetone solution. Although he had from these observations data from which the distribution coefficient of acetone might have been calculated approximately, the results were not considered from this point of view. Indeed near the close of his paper, in discussing the reason for the different rates of exhalation of chloroform, methyl and ethyl alcohol, and acetone, he suggests that the different behavior is determined by their *solubility* in and *miscibility* with plasma, and states that "acetone while completely miscible with water is not so nearly related in constitution and its evaporation and exhalation are thus *less dependent on its concentration in the blood.*" We find, on the contrary, that the rate of evaporation of acetone, at any rate, and its exhalation from the lungs is directly dependent upon its concentration in the blood, which in turn determines the partial pressure in the (alveolar) air with which the solution (blood) is in equilibrium. Acetone in dilute aqueous solutions, in the body as well as *in vitro*, appears to behave in accord with Henry's law and it is perhaps not unlikely that such other substances as chloroform and alcohol would be found to behave in the same way. Cushny, nevertheless, concluded that the "exhalation of volatile substances from the lungs is exactly analogous to their evaporation from solutions in water," which as regards acetone is confirmed by our results.

Finally, in a recent paper Widmark (7) has reported experiments covering almost the identical ground as our own and leading to the same conclusions. He determined the distribution coefficient of acetone between water and air at 38°C. and found the ratio,  $\frac{\text{concentration in solution}^1}{\text{concentration in air}}$  to be 406 and 389 by two different methods. In one case only the concentration in air,

<sup>1</sup> Widmark gives the ratio  $\frac{\text{air}}{\text{solution}}$ , the reciprocal of the relation, which we have preferred to use. We have recalculated his data for comparison with our own.

and in the other only the concentration in solution was determined, the other concentration being calculated or assumed in both cases. For blood serum he gives 392 and for whole beef blood 323 and 304 as values of the same ratio. On making simultaneous analyses of blood and alveolar air of a normal subject after taking various amounts of acetone by mouth, he found as values of  $\frac{\text{concentration in blood}}{\text{concentration in alveolar air}}$  from 394 to 334, thus demonstrat-

ing approximately the same relationship as found *in vitro* with blood. In another paper Widmark (8) showed that the concentration of acetone in urine very closely parallels its concentration in blood and that the amount excreted by the kidneys in a given time thus depends only upon the blood content and the urine volume.

#### *Distribution of Acetone between Water and Air, in Vitro.*

For the determination of the distribution coefficient of acetone we have used several different procedures of which only one need be described in detail. In all cases we have directly determined by the iodoform titration the amount of acetone both in the air and in the solution with which the air was in equilibrium. In one method about 2 liters of air were shaken with about 500 cc. of acetone solution in bottles at room temperature (laboratory, warm room, and cold room), and in another a measured volume of air was bubbled through successive tubes of acetone solution placed in a water thermostat. Observations were made over a considerable range of temperature and with solutions of different concentration.

The arrangement of apparatus usually used is shown in Fig. 1. Tubes, *A*, *B*, and *C* and the bottle, *D*, were nearly filled with the same acetone solution and were covered by the water of the thermostat which was stirred and maintained within 0.1° of the desired temperature. A thermometer in the bottle indicated the temperature at which the air and solution were in final equilibrium, and an additional exit tube was connected with a water manometer (*E*), to insure atmospheric pressure. Air was bubbled through, using just enough pressure at the inlet and suction at the outlet to maintain a steady flow at atmospheric pressure

in the bottle, *D*. Two tubes each containing about 60 cc. of cold water, and surrounded by water and ice, outside the thermostat, absorbed the acetone from the air,<sup>2</sup> the volume of which was measured at room temperature by the water displaced from the siphon bottle, *F*. The volume was calculated to the temperature of the bottle, *D*, at which equilibrium was established, making correction for temperature and water vapor. Usually about 2 liters of air were taken for a determination. Samples of the solution were withdrawn from the bottle, *D*, from time to time and analyzed. The concentration in *D* changes very

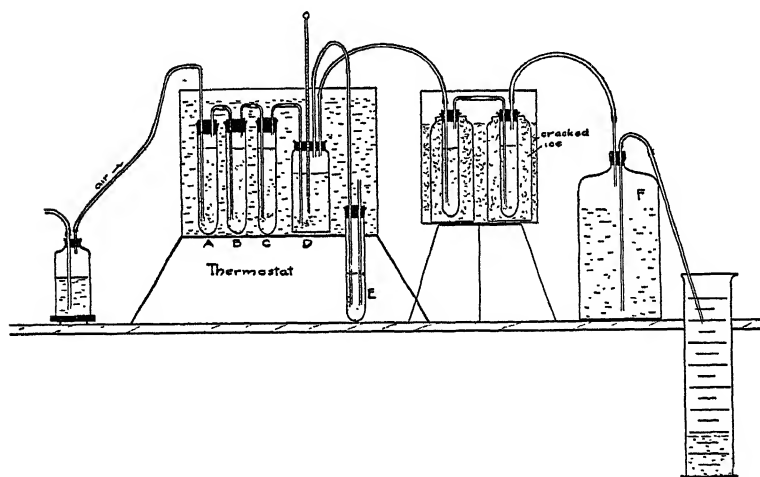


FIG. 1. Apparatus used for determining distribution coefficient of acetone between solution and air.

slowly since the air receives its acetone from the earlier tubes. The following example illustrates the data determined and the method of calculation.

<sup>2</sup> That the acetone was for practical purposes completely absorbed in the two tubes of ice water was proved by the following experiment. 4 liters of air passed through the tubes of the thermostat which contained an acetone solution of approximately 0.1 per cent. The acetone was collected in a series of three tubes of ice water (4.0°C.). The acetone in the first two tubes was determined by titration to be 42.7 mg. The amount of acetone in the third tube was determined nephelometrically to be 0.0118 mg. or about 0.003 mg. per liter of air. The ratio  $\frac{\text{solution}}{\text{air}}$  at 5° is about 1.900.

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Temperature (of solution during equilibration) = 37.8°C. Barometric pressure = 750. 2,000 cc. of air measured at 27.4°C. contained 6.85 mg. of acetone. 10 cc. of acetone solution from the last tube in the thermostat contained 10.64 mg. of acetone, or 1,064 mg. per liter.

(Vapor tension, H<sub>2</sub>O at 27.4° C. = 27 mm. at 37.8° = 49 mm. of Hg)

$$2,000 \times \frac{273 + 37.8}{273 + 27.4} \times \frac{750 - 27}{750 - 49} = 2,134 \text{ cc. at } 37.8^\circ$$

$$\frac{1,000}{2,134} \times 6.85 = 3.21 \text{ mg. of acetone per liter of air.}$$

$$\text{Ratio of concentrations, } \frac{\text{solution}}{\text{air}} = \frac{1,064}{3.21} = 332.$$

The interest being in the values of the distribution coefficient at body temperature, only the results between 37° and 38°C. are given (Table I). These results and many others at higher and lower temperatures show that the ratio between solution and air is quite independent of concentration over a wide range.

The distribution coefficient was determined also for blood serum (beef) with added acetone using an additional tube with glass-wool as a trap to catch foam. It will be seen that the values are practically the same as with pure solutions of acetone.

The average of results give the following values:

$$37\text{--}38^\circ \frac{\text{solution}}{\text{air}} = \text{for pure solutions, } 334; \text{ for serum, } 337.$$

A few determinations were made also with defibrinated blood (beef), but more difficulty was experienced from foaming, and the results were not quite reliable. They indicated roughly the same values as obtained with serum.

*The Ratio of Acetone in Blood and Alveolar Air.*—In order to determine acetone in alveolar air it was desired to have the animals or subjects rebreathe into a bag as in the Plesch method for CO<sub>2</sub>. A rubber bag was found unsuitable due to a rapid loss of acetone vapor, probably by solution in the rubber. The concentration of acetone in the air decreased about 30 per cent in a half hour.

A satisfactory bag was made from sheets of adhesive plaster, to the gummed side of which glazed tracing paper was attached. Two sheets of this fabric 8 by 10 inches were glued and clamped together along three sides; into the open end of the bag thus formed, a covered rubber stopper carrying a glass outlet tube was fitted and glued in place. In order to absorb moisture from expired

air, and prevent its condensation inside the bag, a short calcium chloride tube was attached to the outlet tube of the bag, fresh lumps of  $\text{CaCl}_2$  being used for each experiment. When air was bubbled through an acetone solution of known concentration in the thermostat apparatus into this bag and subsequently drawn from the bag through ice water, the amount of acetone

TABLE I.

*Distribution of Acetone between Solutions and Air.*

Temperature.	Acetone per liter.		Ratio.
	Solution.	Air.	$\frac{\text{Solution}}{\text{Air}}$
Acetone in distilled water.			
	mg.	mg.	
37.0	2, 143	6.65	323
37.1	2, 143	6.74	318
37.7	1, 220	3.67	332
37.8	1, 120	3.43	328
37.2	1, 115	3.29	339
37.3	255	0.748	341
37.3	1, 064	3.07	347
37.5	1, 064	3.08	346
37.9	1, 064	3.20	333
37.8	1, 064	3.23	329
37.8	5, 326	16.06	332
Average.....37.50			334
Acetone in blood serum.			
37.6	3, 020	8.69	347
37.0	3, 020	9.22	328
37.6	3, 020	9.12	332
37.4	3, 020	8.99	336
Average.....37.4			337

obtained from a measured volume was in agreement with that computed from the concentration of the solution and the distribution coefficient at the observed temperature. With this bag, thus shown to be relatively impermeable to acetone vapor, we determined the concentration of acetone in the alveolar air of a number of human subjects and of experimental animals.



After rebreathing from the bag for a period of 30 to 60 seconds, the air was passed through two tubes of ice water, surrounded by water and ice, the volume of air being measured as in the thermostat experiments at room temperature and calculated to its volume, moist, at body temperature. The acetone absorbed by the tubes of ice water was determined by titration with 0.01 or 0.002 N iodine and thiosulfate.

The determination of acetone in blood was accomplished by aerating the Folin-Wu blood filtrates. Oxalated blood was precipitated by tungstic acid according to the Folin-Wu directions (9), using small flasks to minimize the volume of air in contact with the solution. The mixtures were filtered on funnels with long stems, and covered by watch-glasses to minimize loss during filtration. 10 cc. of the filtrate, equivalent to 1 cc. of blood, were measured into large test-tubes containing about 5.0 gm. of NaCl, and the solution was aerated into another large test-tube containing 50 cc. of water, 2 cc. of 5 N NaOH, and 10 cc. of 0.002 N iodine. After 10 minutes of slow aeration, an additional 10 cc. of iodine were added, and the rate of aeration increased and continued 15 minutes. Normal blood to which were added known amounts of acetone, when analyzed under these conditions, gave results within 2 or 3 per cent of the calculated value.<sup>3</sup>

### *Experiments on Dogs after Injection of Acetone Solutions.*

*Experiment 1.*<sup>4</sup>—A female dog of 7.5 kilos was given 4 gm. of urethane in 200 cc. of water by mouth. A tracheal cannula was inserted under local anesthesia (benzyl alcohol). The bladder was emptied by catheter and control samples of blood and alveolar air were taken, after which 75 cc. of 14.5 per cent acetone solution (10.87 gm. or 1.45 gm. per kilo of body weight) were injected intravenously. Samples of blood, alveolar air, and urine were collected as stated in the protocol.

10.21 a.m. Control air, 760 cc. at 28° = 805 cc. at 37.6° = 0.046 mg. of acetone. Control urine, 13 cc. 10.24 a.m. Bladder emptied. 10.27 a.m. Blood control. 10.50 a.m. 75 cc. of 15 per cent acetone solution intravenously. 10.52 a.m. Urine 1 = 3.3 cc. 10.59 a.m. Air 1 = 680 cc. at

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<sup>3</sup>Air under pressure was used in aeration. After 30 minutes aeration, 20 cc. of 0.01 N iodine showed a loss of about 0.15 cc. and 20 cc. of 0.002 N iodine a loss of about 0.25 cc.

<sup>4</sup>The thanks of the authors are due Dr. E. K. Marshall, Jr., and Dr. W. H. Olmsted for assistance in conducting several of the experiments.

28° = 720 cc. at 37.6° = 5.02 mg. of acetone. 11.00 a.m. Blood 1 = 15 cc. 11.06 a.m. Urine 2 = 5.2 cc. + 20 cc. of wash water. 11.11 a.m. Rectal temperature = 37.6°. 11.15 a.m. Air 2 = 650 cc. at 28° = 689 cc. at 37.6° = 4.41 mg. of acetone. 11.24 a.m. Blood 2. 11.28 a.m. Air 3 = 660 cc. at 28° = 700 cc. at 37.6° = 4.07 mg. of acetone. 11.33 a.m. Blood 3. 11.35 a.m. Urine 3 = 21 cc. + 20 cc. of wash water. 11.45 a.m. Air 4 = 730 cc. at 28° = 774 cc. at 37.6° = 4.38 mg. of acetone. 11.48 a.m. Blood 4. 11.58 a.m. Urine 4 = 10 cc. + 20 cc. of wash water. 12.02 p.m. Air 5 = 650 cc. at 28° = 689 cc. at 37.6° = 3.63 mg. of acetone. 12.06 p.m. Blood 5. 12.14 p.m. Urine 5 = 6.5 cc. + 20 cc. of wash water. 12.28 p.m. Air 6 = 750 cc. at 28° = 795 cc. at 37.6° = 3.84 mg. of acetone. 12.30 p.m. Blood 6. 12.37 p.m. Urine 6 = 3.5 cc. + 20 cc. of wash water. 12.56 p.m. Blood 7. 1.00 p.m. Air 7 = 695 cc. at 28° = 737 cc. at 37.6° = 3.62 mg. of acetone. 1.06 p.m. Urine 7 = 6 cc. 2.01 p.m. Blood 8. Air 8 = 688 cc. at 28° = 729 cc. at 37.6° = 3.39 mg. of acetone. 2.02 p.m. Urine 8 = 5.8 cc.

The analytical results of this experiment are given in Table II and have been plotted in time curves shown in Fig. 2. For the calculation of the ratios between concentration of acetone in blood and in alveolar air, given in the last column of the table, a curve of the concentration in blood plasma was plotted and values (in parentheses in table) read off corresponding to the time of taking the air samples. The ratio of concentrations  $\frac{\text{plasma}}{\text{air}}$  varies from 320 to 350, the average being 336, or nearly

the same as observed in the *in vitro* experiments (337). The variation is irregular and is doubtless due to accumulated errors in the procedure. The curves show that the concentration of acetone in both alveolar air and urine follows very closely that in blood, the amount in urine and blood being almost identical. The initial rise in the urine is probably in response to a much higher concentration in the blood immediately after the injection and before the first blood sample was drawn. With the fall of the amount in blood due in large part to its passage into the tissues, the concentration in urine fell to or slightly below that in blood.

It is of interest to learn the relative amounts of acetone excreted by this animal from the lungs and kidneys. The total urine secreted during 3 hours and 10 minutes was 61 cc., containing 114 mg. of acetone. The total excretion by the lungs was

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not determined but may be very roughly calculated as follows. The respirations varied from 13 to 25 per minute of from 100 to 130 cc., giving a total volume per minute of  $18 \times 115 = 2,070$  cc. The alveolar air may be taken as containing about 5 mg. per liter and the tidal air perhaps 4.0 mg. per liter. This gives a total excretion by the lungs during the experiment of 1.56

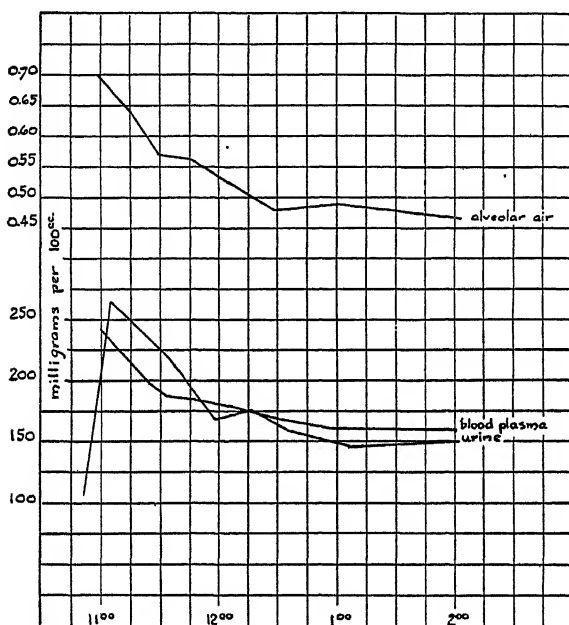


FIG. 2. Curves showing parallel changes in concentration of acetone in blood, alveolar air, and urine of dog after intravenous injection (Experiment 1).

gm. or roughly fifteen times the excretion by the kidneys during the same time. Since the concentration in urine is independent of the urine volume, the amount thus excreted will, of course, depend upon the amount of urine secreted as well as upon the blood concentration. And similarly the amount excreted in a given time by the lungs is determined by the volume of respired air which aerates the acetone from the blood.

TABLE II.  
*Experiment 1.*

Time.	Acetone per 100 cc.				Ratio.
	Whole blood aerated.	Blood plasma aerated.	Urine.	Alveolar air.	Blood plasma Alveolar air
<i>a. m.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.21	1.8			0.0057	
10.27					
10.50	Acetone injected.				
10.52			106		
10.59		(242)		0.698	347
11.00	236	242			
11.06			264		
11.15		(205)		0.640	320
11.24	198	196			
11.28		(194)		0.571	340
11.33	188	191			
11.35			217		
11.45		(185)		0.566	327
11.48	186	184			
11.58			168		
<i>p. m.</i>					
12.02		(179)		0.527	340
12.06	177	176			
12.14			175		
12.28		(170)		0.483	350
12.30	168	169			
12.37			159		
12.56	165	161			
1.00		(160)		0.491	326
1.06			147		
2.01	163	159		0.465	342
2.02			151		
Average .....					336

*Experiment 2.*—Under ether anesthesia a tracheal cannula was inserted in a dog weighing 10 kilos. Urethane was injected intraperitoneally and the ether discontinued. Blood and air samples were taken before and after the injection of acetone solution as stated in the protocol. The analytical results are given in Table III.

2.45 p.m. 10 gm. of urethane intraperitoneally. 3.00 p.m. Control samples of blood and alveolar air. 3.05 p.m. 25 cc. of acetone solution

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(50 per cent) injected into the peritoneal cavity. 3.23 p.m. Blood sample 1. 3.27 p.m. Air sample 1 = 395 cc. at 27°. 3.55 p.m. Blood sample 2. 4.00 p.m. Air sample 2 = 410 cc. at 27°. 4.10 p.m. Blood sample 3. 4.14 p.m. Air sample 3 = 330 cc. Rectal Temperature 97°. 4.25 p.m. Blood sample 4. 4.30 p.m. Air sample 4 = 400 cc. at 27°. 4.40 p.m. Blood sample 5. 4.43 p.m. Air sample 5 = 295 cc. at 27°. 5.00 p.m. Blood sample 6. 5.05 p.m. Air sample 6 = 380 cc. at 27°. 5.15 p.m. Blood sample 7. 5.25 p.m. Air sample 7 = 520 cc. at 27°. 5.35 p.m. Blood sample 8. 5.40 p.m. Air sample 8 = 250 cc. Rectal Temperature = 97°. Dog killed with chloroform.

TABLE III.  
*Experiment 2.*

Time.	Acetone per 100 cc.		Ratio.
	Blood serum.	Alveolar air.	
<i>p. m.</i>	Acetone solution injected (25 cc. of 50 per cent).		$\frac{\text{Concentration of serum}}{\text{Concentration of alveolar air}}$
3.05	157		
3.23	(156)	0.453	344
3.27	149		
3.55	(145)	0.471	307
4.00	132		
4.10	(131)	0.390	336
4.14	128		
4.25	(127)	0.392	324
4.30	125		
4.40	(125)	0.377	329
4.43	123		
5.00		0.328	369
5.05	114		
5.15		0.368	310
5.25	113		
5.35		0.357	317
5.40			
Average.....			330

The ratios  $\frac{\text{serum}}{\text{alveolar air}}$  vary considerably, from 307 to 369, the average being 330. It will be noted that the acetone (12.5 or 0.8 gm. per kilo) was injected into the peritoneal cavity from which it rapidly passed into the blood and from the blood was more slowly distributed and excreted.

Four other experiments of similar character yielded results which gave ratios,  $\frac{\text{blood}}{\text{alveolar air}}$ , from about 280 to 380, most of which were of doubtful accuracy and for that reason will not be recorded. The acceptable results leave no doubt that distribution of acetone between blood and air in the lungs of dogs is substantially the same as the distribution found *in vitro*.

*Distribution of Acetone between Blood and Alveolar Air in Diabetic Acidosis.*

The determinations in blood and alveolar air were carried out as above described, at once after collecting samples. The results obtained are given in Table IV in which is also included for comparison a summary of the results on animals and a few of the results of *in vitro* experiments. Results are recorded also of two determinations on normal subjects on the morning of the third day of fast, when acetone bodies were being formed in small amounts. For the determinations of acetone in alveolar air of these normal subjects 5 liters of air, separately equilibrated by rebreathing 1 liter portions, were taken for analysis. The ratio between blood and air was evidently the same as in the diabetic subjects with marked acidosis.

These results from diabetic and normal subjects show conclusively that the concentration of acetone in alveolar air bears a constant relationship to the concentration of free, preformed acetone in the blood, and that this relationship is expressed by the distribution coefficient of acetone between the air and its solution in blood plasma. From this fact it follows that one may learn the amount of acetone in blood by determining the amount in alveolar air and multiplying the result by a factor, the value of which according to our data is about 340. And if there were also a constant relation between the amount of acetone, and of the related acetoacetic and hydroxybutyric acids it would be possible to calculate also the latter values. In Table V are given the results on a few blood analyses showing separately the amounts of acetone, and acetoacetic and hydroxybutyric acids.

Although these results are too few to justify generalization, they indicate that the relative amounts of free acetone in blood

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vary at least from 13 to 26 per cent of the sum of acetone, and acetoacetic and hydroxybutyric acids. One may, therefore, perhaps get a rough approximation of the amount of total acetone bodies (expressed as acetone) present in blood by multiplying the concentration in alveolar air by, say 1,700, ( $340 \times \frac{1.00}{2.0}$ ). Such a calculation is not to be recommended as a substitute for

TABLE IV.

*Summary of Results, Ratio of Acetone in Blood and Alveolar Air, and Comparison with Results in Vitro.*

Subject.	Condition.	Acetone per liter.		Ratio
		Blood.	Alveolar air.	$\frac{\text{Blood}}{\text{Air}}$
		Whole blood. <i>mg.</i>		
P. R.	Diabetic acidosis.	112	0.324	345
O. L.	“ “	96	0.278	345
F. M.	“ “	148	0.436	340
Peters.	“ “	199	0.560	355
K.	Normal, 3 days of fast.	64	0.163	392
C.	“ 3 “ “ “	23	0.066	350
	Acetone injection.	Blood plasma.		
Dog.	Experiment 1.	2,420 to 1,590	6.98 to 4.65	336
“	“ 2.	1,570 to 1,130	4.53 to 3.57	330

*In vitro* experiments.

Water solutions.	Solution.	Air.	$\frac{\text{Solution}}{\text{Air}}$
Maximum concentration. ....	5,326	16.06	332
Minimum “ .....	255	0.748	341
Average.....			334
Blood serum.....	3,020	8.96	337

the direct determination in blood. The excretion of acetone by the lungs is nevertheless of real value as affording a very simple means of detecting and roughly determining the extent of ketosis. The subject is asked merely to exhale for 1 or 2 minutes through a glass tube into a large test-tube of ice cold distilled water. At the end of the period of exhalation 10 or 20 cc. of Scott Wilson reagent are added and if ketosis exists a faint to deep opalescence

or precipitate appears after a few moments, the amount being roughly proportional to the amount of acetone exhaled. This test is quite sensitive and appears even before the urine shows a positive reaction with ferric chloride for acetoacetic acid.

TABLE V.  
*Acetone Bodies in Blood.*

No.	Subject.	As acetone in 100 cc. of blood.				Per cent of total.				Total acetone bodies in 24 hrs. as acetone in urine.	Remarks.
		Acetone.	Acetoacetic acid.	Hydroxybutyric acid.	Total.	Acetone.	Acetoacetic acid.	Hydroxy butyric acid.			
									gm		
1*	"K," Severe diabetic.	17.6	10.4	45.2	73.2	24	14	62	56.7		Not in coma.
2*	" " "	3.3	7.7	13.5	24.5	14	31	55	15.9		" " "
3*	" " "	11.0	7.0	49.5	67.4	16	10	74	22.1		" " "
4*	Infant, 2 yrs.	3.8	9.5	15.8	29.1	13	33	54			" " " pyelitis.
5	"P," Severe diabetic.	19.9	15.6	42.5	78.0	25	20	55	27.1		Not in coma.
6	" " "				100.0			37			Death, not in coma, few hours later.
7	"W" Severe diabetic.	55.6	39.0	184.0	278.0	20	14	66	41.1		Death in coma, same day.
8	"S" Severe diabetic.				77.0			46	6.7		In light coma.
9	" " "				136.0			40	11.2		Death next day.

\* These results were previously reported from this laboratory by Marriott (Marriott, W. McK., *J. Biol. Chem.*, 1914, xviii, 515).

#### SUMMARY AND CONCLUSIONS.

1. The distribution coefficient of acetone for water and air in the vicinity of 37° and 750 mm. has been determined by two methods and found to be about 334 for  $\frac{\text{water}}{\text{air}}$ .



By the same technique the distribution coefficient of acetone for blood serum and air was found to be about 337.

2. The distribution of acetone in whole blood, blood plasma, blood serum, urine, and alveolar air of dogs which had been injected with large doses has been determined. The results show that the concentration of acetone in urine is about the same as that of whole blood and blood plasma and that the ratio of acetone in blood to that in alveolar air is about 333.

The distribution of acetone between alveolar air and blood of human diabetics and of normal fasting subjects was also determined and found to average 355.

From the above data it is concluded that acetone is excreted from the lungs and kidneys by the physical process of diffusion, thus confirming the recent observations of Widmark.

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# CARBOHYDRATE CONTENT OF THE KING SALMON TISSUES DURING THE SPAWNING MIGRATION.\*

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The facts reported in this paper were developed as a part of the series of physiological studies on the king salmon, *Oncorhynchus tshawytscha*, during the summer of 1906. This salmon feeds in the California coastal waters to maturity and then migrates through San Francisco Bay and up the Sacramento River to spawning beds in the cold snow-fed waters of the McCloud River on the slopes of Mt. Shasta. The journey requires from 60 to over 100 days and is made without food.

The source of the energy used in salmon migration has long been a physiological problem of peculiar interest. Miescher studied the migrating Rhine salmon. Paton and others have published extensive papers on the chemical changes in the Scottish salmon, but carbohydrate metabolism has received little or no consideration.

It is not known to what extent the carbohydrates enter in the processes which liberate the dynamic energy expended in migration. The king salmon stores enormous percentages of fat, as in fact do all the salmon species thus far examined. This fat disappears during the migration and is probably the chief source of energy.<sup>1</sup> Kilborn and Macleod<sup>2</sup> have recently given analytical data to show that the tissues of certain marine invertebrates and fishes are low in glycogen, or its equivalent carbo-

\* Published by permission of the United States Commissioner of Fish and Fisheries.

<sup>1</sup> Greene, C. W., *Bull. U. S. Bureau Fisheries*, 1913, xxxiii, 69.

<sup>2</sup> Kilborn, L. G., and Macleod, J. J. R., *Quart. J. Exp. Physiol.*, 1918-20, xii, 317.

hydrate. In the single salmonoid reported, the lake trout, he found only a trace of carbohydrate in the muscle tissue.<sup>3</sup>

Our studies were made to supplement the general chemical studies by measurement of the glucose content of the tissues of the king salmon at different stages of the migration. Granting that the kinetic energy expended during the migration is derived primarily from the oxidations of the fats, still carbohydrates might play a significant part. Also, the developing ovaries store large amounts of food material in the yolk (the mature salmon egg is 6 to 7 mm. in diameter, hence contains 113 or more c.mm. of yolk). Carbohydrate is often present in the yolk of other eggs and should be found in the salmon eggs, at least when they are mature at the spawning.

Analyses were made of the following tissues: muscle, liver, ovary and ripe eggs, testes, skin, and stomach-intestine. Fishes of the series were chosen from the feeding grounds at Monterey Bay and Bolinas Bay,<sup>4</sup> Black Diamond, at the head of tide-water at the entrance of the Sacramento River into San Francisco Bay,<sup>5</sup> and at Baird on the spawning grounds on the McCloud River in the mountains of northern California.

### *Method.*

The tissues or organs were ground fine in a meat chopper, the samples were weighed and preserved in 95 per cent alcohol in wide mouthed 240 cc. glass-stoppered bottles. The samples not immediately analyzed were sealed and shipped to the chemical laboratories at Stanford University.<sup>6</sup> The concentration of alcohol was that used in preserving other analytical samples which have been demonstrated to undergo no autolytic change. The samples

<sup>3</sup> The first announcement of the present data was made by the author during the discussion of the preliminary paper of Dr. Macleod presented at the Cincinnati meeting of the American Physiological Society, December 29, 1919.

<sup>4</sup> These fish are considered as the nearest representatives of the fully mature salmon entering the Golden Gate.

<sup>5</sup> No glucose analyses were obtained of samples from this station.

<sup>6</sup> The writer is under deepest obligation to Professor Lyonel R. Lenox of Stanford University for his cooperation and assistance in securing the chemical determinations.

TABLE I.  
*Percentage of Glucose in Salmon Tissues of Fish Taken at Different Stages of Migration.*

Station.	Date.	Fish No. and sex.	Total length. mm.	Weight of salmon. gm.	Muscle. per cent	Liver. per cent	Ovary. per cent	Testes. per cent	Skin. per cent	Stomach. per cent
Monterey Bay.	1906 June 15	623 ♀	876	9,700		0.39				
	" 23	690 ♂	820	7,200		0.61				
	" 26	691 ♂	898	10,650		0.32				
	" 29	694 ♂	1,000	14,334		0.66				00
	July 18	725 ♀	860	10,000		0.19				
Bolinas Bay.	" 20	732 ♀	867	9,050	0.015 0.014	0.18 0.26	0.18			Trace.
	July 26	754 ♀	906	10,700	Trace.	0.70	0.09			
	" 26	765 ♂	890	11,350	" "	0.21 0.18			0.038	0.041
	Aug. 15	810 ♀	745	5,200	00	0.08	0.072 0.069			
	" 15	821 ♀	814	6,650	00 00		0.08 0.09		00	
McCloud River.	" 16	850 ♂	1,046	9,350	00	0.123	00	00	00	
	" 28	938 ♀	915	8,450	00	0.026	0.09			
	" 28	939 ♂	875	7,300	00	0.021	0.07			
	" 28	940 ♂	890	6,300	00	0.054 0.110		00	00	
	" 30	946 ♀	878	7,650	00	0.017				
	" 4	975* ♂	948	9,800	00	0.020 0.019	0.09, eggs.	00		
					00					

\* Fine, dark-colored, immature, and pink meated fish.

were analyzed by the method of Pfüger and the glycogen was hydrolyzed and determined as glucose by the copper sulfate colorimetric method. The hydrolysis was used to guard against any loss by possible autolysis occurring during the preservation. The determinations are comparatively few but we have had no opportunity to add to the series. There are data enough in Table I, which gives the entire set of determinations for the series of samples, to indicate the average glycogen content of salmon tissues and the contrasts at the extremes of the fast.

#### *Muscle.*

Samples were taken of the great lateral muscle in a vertical band in the region of the anterior dorsal fin. In Fish 732, Monterey Bay, July 18, the duplicate samples show 0.015 and 0.016 per cent of glucose. These are voraciously feeding fish and are to be contrasted with Fishes 754 and 765 from Bolinus Bay near the mouth of the Golden Gate. The Bolinus fish are in the very prime of condition with the high store of 18 per cent of muscle fats.<sup>7</sup> They represent the highest nutritive value of any fish in this series, greater than the average for feeding fish at Monterey and of higher value than the migrating fish at tide-water on the Sacramento River. Fish at Bolinus Bay at this time had practically ceased feeding (judged by the absence of food content in the stomach).

Muscle samples were not obtained for glucose analysis from the Black Diamond fish.

The muscles of spawning fish are without glucose as shown by the analyses from four females and three males. Not a single sample yielded glucose. The tissues were perfectly fresh, often alive when minced.

#### *Liver.*

The salmon livers in fish from the Monterey feeding grounds show a variable glucose content, from 0.19 to 0.66 per cent. Livers from these fish examined histologically were crowded with fat droplets. Chemical analyses showed fat as high as 16.6 per cent. The glucose content is from ten to forty times greater than that

<sup>7</sup> Greene, C. W., *Tr. Am. Fisheries Soc.*, December, 1915.

of the muscle in the Monterey fish. The two Bolinus Bay fish vary as much as the extremes of the six examined at Monterey; *i.e.*, 0.18 and 0.70 per cent of glucose.

Samples of livers from six fish were analyzed from Baird. These livers from spawning fish contain with two exceptions less than 0.1 per cent of glycogen. The average is 0.057 per cent. This average is strikingly lower than the glycogen content of the Monterey or Bolinus Bay fishes. It indicates a depletion of the glycogen and a decrease of the part played by carbohydrate in liver metabolism during the fast.

#### *Ovaries and Eggs.*

The ovaries of the series of six fish are remarkably uniform in glucose content, whatever the stages of the journey. The percentages are from 0.08 to 0.18. This is a glucose content not above the average found in vertebrate blood.<sup>8</sup> One fish, No. 946, was a mature female from which spawning eggs were obtained. The analysis of these eggs, free from ovarian tissue and fluids, gave 0.09 per cent of glucose. This is near the average for the ovary of the series of immature fish. It demonstrates that the carbohydrate content in this growing tissue is independent of the stage of development and of the duration of the fast.

Tichomiroff<sup>9</sup> has given analyses of invertebrate eggs showing that in the eggs of Bombyx the glycogen amounts to 1.98 per cent. The total dry substance in these eggs is 35.51 per cent, somewhat less than salmon eggs, which average 45 per cent or more in total solids. Octopus eggs have been examined by Henze,<sup>10</sup> who found as high as 1 per cent of glucose in the fresh eggs (5.4 per cent in the dry residue). Kojo<sup>11</sup> found 0.272 per cent of glucose in the yolk and 0.55 per cent in the whites of the hen's egg.

#### *Testes.*

Three samples of testes were examined for glucose from the Baird salmon. The salmon were nearly mature but not one contained glucose.

<sup>8</sup> Macleod, J. J. R., *Physiol. Rev.*, 1921, i, 208.

<sup>9</sup> Tichomiroff, A., *Z. physiol. Chem.*, 1885, ix, 566.

<sup>10</sup> Henze, M., *Z. physiol. Chem.*, 1908, lv, 433.

<sup>11</sup> Kojo, K., *Z. physiol. Chem.*, 1911, lxxv, 1.

*Other Organs.*

Samples of the skin and of the stomach-intestinal mass were analyzed. The skin sample of Fish 765 from Bolinus Bay contained 0.038 per cent of glucose. Three samples of skin from spawning fish contained no glucose.

Of the three stomach-intestinal samples, two were from Monterey. One gave a trace of glucose, the other none. The specimen from Bolinus Bay contained 0.041 per cent of glucose. However, the pyloric ceca and the intestine of both Monterey and Bolinus Bay fish contained a variable quantity of mucus and unabsorbed food products. These are retained in part in the ground up total mass used for the sample, hence the 0.041 per cent of glucose from the Bolinus Bay specimen might have come from the food remnants, though it is improbable.

The stomach and intestines of McCloud River fish are greatly atrophied and small. However, no glucose analyses were obtained.

## DISCUSSION.

Kilborn and Macleod<sup>2</sup> have emphasized the comparatively low content of glycogen in invertebrates and fishes. The four fish species examined by them were dogfish, chimæra, carp, and lake trout. In their Table IV they present analyses of muscle, heart, and liver of dogfish, of carp, and of trout and give determinations for the liver of the chimæra. In the dogfish their average for body muscle is 0.018 per cent, for carp it is 0.29 per cent, and in lake trout muscle there is only a trace of glucose. Our analyses of the sea run salmon check against these determinations, 0.015 per cent in the salmon muscle in comparison with 0.018 in the dogfish and 0.29 in the carp.

On the other hand, the liver determinations of Kilborn and Macleod show as much as 5.6 per cent of glucose in the carp liver but only a maximum of 0.16 per cent for dogfish liver and 0.055 per cent for the liver of lake trout. The lake trout figure checks with that of our fasting salmon. They find a trace of glucose in lake trout muscle (probably fasting) while we find none in the fasting salmon. They have presented no analyses of the glucose content of either eggs or ovaries of fishes. In fact we have not been able to find such in the literature.

No previous comparisons have been made of the glucose content of tissues during long starvation, comparisons for which the salmon migratory habit without food lends a rare opportunity. A study of our data will show that the muscle glucose, presumably glycogen, is present in low amount during the feeding period, but drops to a trace at the beginning, and disappears entirely during the migration. Since it has been shown in several of my earlier articles<sup>1</sup> that the large store of fats progressively decreases with the migration, one can scarcely escape the view that the carbohydrates play little part in supplying kinetic energy for the migration; in fact the small amount of glucose in the muscles of feeding salmon may well be present by virtue of the digestive and anabolic processes going on at that time.

That glycogen is not entirely absent in salmon metabolism during the migratory journey is indicated by its constant presence, though in small amount, in the liver and ovaries. In view of the well known liver glycogenic function in vertebrates, and especially in mammals, it is surprising to find so small a percentage present in the salmon liver even under the most favorable conditions of feeding. In our determinations it has never exceeded 0.70 per cent of the wet weight of the organ. Livers from the Monterey feeding fish, in which the glycogen reaches its highest amount, invariably contain a considerable percentage of fat. For example, in the liver of No. 765 from Bolinus Bay unpublished analyses reveal as much as 25.8 per cent of fat, which is the highest liver fat observed. In general the normally small percentage of glycogen is greatly diminished in the spawning salmon. If we contrast the Monterey fish with the Baird spawning fish the averages for the liver are 0.405 and 0.057, respectively.

We are most pleased by the discovery of the constant composition of the ovaries in glucose (glycogen). The average for the entire series is 0.096 per cent. It seems to us to point directly to a uniform synthetic and storage process in this tissue, removed as it is from any part in energy production during the migration, but constantly growing and actively storing foods. This uniformity of composition as regards glucose adds one more point in evidence to the unpublished data of the author showing uniformity of composition of the food-loaded protoplasm of the egg cell at whatever stage of its growth it be considered.



## 436 Carbohydrate of King Salmon Tissues

The protein and fat percentages of fish from this series were published in 1915.<sup>7</sup> We reproduce the averages of the table in that paper but with columns for glucose introduced. Unfortunately we have no glucose determinations for tide-water fish. Both proteins and fats are stored in the mature salmon muscle in quite large excess. This storage of proteins and fats is also true for salmon ovaries.<sup>12</sup> The muscle protein excess is 6 per cent of the tissue figured on the protoplasmic basis. The fat is stored

TABLE II.

<sup>1</sup> Protein, fat, and glucose in wet muscle samples giving average percentages for Monterey Bay, tide-water on the Sacramento River, and the spawning beds on the McCloud River.

Station.	Protein.*	Fat.*	Glucose.	Glucose in liver.	Glucose in ovary.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Monterey Bay.....	15.6	18.0	0.015	0.606	0.13
Tide-water.....	16.9	14.6			
McCloud River.....	14.4	1.6	0.000	0.130	0.09

\* Protein and fat taken from Greene's<sup>7</sup> table.

to 18 and more per cent of the moist tissue (25 to 30 per cent of the dark muscle). No such large storage of carbohydrate occurs in any salmon tissue. Carbohydrate is never present in more than 0.70 per cent even in the livers of feeding salmon.

Carbohydrate is always present in the growing ovary, is in small amount in all the tissues of the feeding salmon, but disappears from the muscles and drops to a lower level in the liver during the migratory fast.

<sup>12</sup> Greene, C. W., *J. Biol. Chem.*, 1921, xlviii, 59.

## VITAMINE REQUIREMENTS OF CERTAIN YEASTS AND BACTERIA.

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(Received for publication, July 30, 1921.)

In a previous publication (1) we described a practical method for testing the vitamine requirements of yeast, based upon the work of Ide and his coworkers, and also of Williams, of Bachmann, and of Eddy. At that time we thought our test was specific for antiberi-beri vitamine; however, because of certain differences, the question was left open.

In this connection, Emmett and Stockholm (2) suggested that the vitamine necessary for the growth of yeast had nothing to do with antiberi-beri vitamine. Later Fulmer, Nelson, and Sherwood (3), Souza and McCollum (4), and MacDonald and McCollum (5), claimed that by improving the medium, results could be obtained similar to those noted after vitamine addition. These claims have been disproved by the work of Eddy, Heft, Stevenson, and Johnson (6), and our own findings are in accord with their conclusions in this particular.

Our present results show rather conclusively that yeast requires for growth a different substance than that needed by animals, since we were able to separate from autolyzed yeast one substance active for yeast and another for rats and pigeons. This separation will enable us to study each substance individually. At the same time, in agreement with our findings, Ide (7) shows that by improving the medium one can actually obtain a slightly better growth, not to be compared in magnitude, however, with the action of the specific vitamine-like substance.

Although the work thus far does not shed immediate light on the test for the antiberi-beri vitamine, it is of importance in the study of the vitamine requirements of yeasts and bacteria. We believe that we are dealing here with a specific substance—either

a new vitamine or a cleavage product of antiberi-beri, or vitamine B. We wish to point out that although our yeast test previously described does not specifically indicate the vitamine B activity, still it does show, to a certain extent, the relative richness in water-soluble vitamins when we are concerned with naturally occurring foodstuffs.

Simultaneous experiments with yeast and a strain of streptococcus obtained from Mueller (8), and following his method of testing, tend to show a close, if not fully established relationship between their nutritive requirements. It would be very attractive to consider these nutritive elements as one substance, but although there are many points in common, there are still many differences to be reconciled, so that the question must be left open for the present.<sup>1</sup>

Another thing worthy of note is that different strains of yeast behave differently as regards vitamine requirement. Some of them, as shown by Nelson, Fulmer, and Cessna (9), seem to be able to synthesize their own vitamine, the initial inoculation providing the first impulse, while others require the addition of extra vitamine.<sup>2</sup>

This difference in the vitamine requirements of various strains of yeast may shed some light on the ultimate physiology of yeast cells. These lower organisms having greater synthetic power appear to be able to utilize the simplest type of vitamine, so that chemically it might be advantageous to study the structure of vitamins in this way.

#### EXPERIMENTAL.

*Differentiation between the Substance Active for Yeast and That Active for Pigeons and Rats.*—In our previous paper, we showed that much larger quantities of fullers' earth were necessary to remove from autolyzed yeast the substance necessary for the

<sup>1</sup> Detailed experiments along this line are being conducted in our laboratory by L. Freedman, and the results will be presented in a later publication.

<sup>2</sup> Peters (10) believed at first that protozoa can live and divide on purely inorganic material, but he found subsequently that they became smaller and smaller and appeared to live at the expense of their own protoplasm; he thinks, therefore, that addition of vitamins is necessary for proper growth.

growth of yeast, than those which are known to be sufficient for the removal of vitamine B; this is in agreement with the findings of Emmett and Stockholm (2). Our present results show that the substance active for yeast may be removed almost quantitatively from autolyzed yeast by two successive shakings each with

TABLE I.

No.	Substance tested.*	Net yeast activity.	Animal activity.
		mm.	
1	Autolyzed yeast.....	14.5	Positive.
2	“ “ shaken with fullers' earth (50 gm. per liter).....	12.0	Negative.
2a	Fullers' earth from No. 2 decomposed with baryta.....	4.0	Positive.
3	Autolyzed yeast (filtrate from No. 2) shaken with fullers' earth (100 gm. per liter).....	6.0	Negative.
3a	Fullers' earth from No. 3 decomposed with baryta.....	3.5	“
4	Autolyzed yeast (filtrate from No. 3) shaken with fullers' earth (100 gm. per liter).....	0.5	“
4a	Fullers' earth from No. 4 decomposed with baryta.....	0	“
5	Autolyzed yeast shaken with norit (50 gm. per liter).....	13.5	“
5a	Norit from No. 5 decomposed with glacial acetic acid.....	3.0	Positive.
6	Autolyzed yeast (filtrate from No. 5) shaken with norit (100 gm. per liter).....	3.0	Negative.
6a	Norit from No. 6 decomposed with glacial acetic acid.....	3.0	“
7	Autolyzed yeast (filtrate from No. 6) shaken with norit (100 gm. per liter).....	0.5	“
7a	Norit from No. 7 decomposed with glacial acetic acid.....	0	“

\* In each case the amount tested was 0.05 cc., so that the results are quite comparable.

100 gm. of fullers' earth or of norit, per liter. It is essential that with every lot of autolyzed yeast, controls must be run to determine the degree of separation. The fullers' earth and norit were decomposed with baryta and glacial acetic acid respectively, according to the method of Seidell, and of Eddy and coworkers. Norit, extracted with baryta, did not yield any active substance.

The various fractions were also tested on rats and pigeons with concordant results, both preventive and curative experiments being performed.

*Specificity.*—The following experiment shows that by improving the medium either in its inorganic or organic moiety (glucose, proteins) the growth of yeast cells can sometimes be improved; however, the magnitude of the resulting response is of an entirely different order than that obtained by vitamine addition. Using the inorganic medium of Fulmer, Nelson, and Sherwood (Medium F) we did not obtain any more growth than on our Nägeli solution. Using Medium F and autolyzed yeast we had even lower results than with Nägeli solution. This corroborates the finding of Eddy, Heft, Stevenson, and Johnson (6).

No.	Medium.	Yeast activity.	Net activity.
		<i>mm.</i>	<i>mm.</i>
1	Blank determination (Medium F).....	3.0	
2	“ “ (Nägeli).....	3.0	
3	Medium F plus 0.05 cc. of autolyzed yeast plus yeast suspension.....	12.5	9.5
4	Nägeli plus 0.05 cc. of autolyzed yeast plus yeast suspension.....	14.0	11.0
5	Medium F plus 0.05 cc. of autolyzed yeast.	0	
6	Nägeli plus 0.05 cc. of autolyzed yeast.....	0	

The results with glucose have shown that an addition of the sugar has little or no effect, contrary to the findings of MacDonald and McCollum (5). It seems that the slight effect obtained with glucose can be eliminated by shaking the sugar solution with an adsorbent.

Ground up meat was extracted and autoclaved till a watery extract no longer showed any vitamine activity. The meat was then subjected to hydrochloric acid hydrolysis, neutralized, and tested again. There was a small but definite activity manifested. The same was true of casein and gelatin but not of zein, egg albumin, or serum albumin. These experiments are being continued.

The addition of glucose to the blank determination does not affect the result.

No.	Medium.	Yeast activity.	Net activity.
		<i>mm.</i>	<i>mm.</i>
1	0.05 cc. of autolyzed yeast .....	12.5	10.0
2	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose.....	13.5	11.0
3	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent charcoal (norit). ....	13.5	11.0
4	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent fullers' earth.....	12.5	10.0
5	0.05 cc. of autolyzed yeast plus 1 cc. of 10 per cent glucose shaken with 10 per cent Lloyd's reagent.....	12.5	10.0
6	Blank (without autolyzed yeast).....	2.5	
7	1 cc. of 10 per cent glucose.....	2.5	0

Regarding the activity of bakers' and brewers' yeast we have found, in agreement with Williams (11) that brewers' yeast extract is more potent than bakers' yeast extract in affecting the growth of brewers' yeast. On the other hand, contrary to Williams (11) we have noted that bakers' yeast extract does not stimulate the growth of bakers' yeast as much as does brewers' yeast extract.

Goy (12) claims to have isolated a nitrogen-free acid which stimulates the growth of bacteria and yeast, but a closer examination of his results does not lend support to his claims, the substance actually isolated being entirely inactive.

Preliminary work shows that there are many points of similarity and dissimilarity between the substance stimulating the growth of yeast and that stimulating the growth of streptococcus. We have found for example that undecolorized heart infusion exhibits a very marked yeast growth-stimulating activity, while the decolorized infusion (decolorized with norit) shows only a very negligible activity. Autolyzed brewers' yeast also showed marked stimulating action on the growth of streptococcus.

Peptone added to the medium gives growth both with yeast and with streptococcus. Casein hydrolysate acts slightly on yeast growth and more so on streptococcus. Hydrolysates of some other proteins did not act on yeast but were active for

streptococcus. It is just such discrepancies as these that make it imperative to obtain more data before reaching definite conclusions. The method of separation from vitamine B of the substance stimulating yeast growth, and which we will provisionally call "vitamine D," will facilitate further work and may help to clear up the question of the identity of vitamine D with the substance stimulating the growth of streptococcus.

#### CONCLUSIONS.

We have separated from vitamine B a substance which we shall call provisionally vitamine D and which acts on microorganisms.

Vitamine D appears to be a definite and specific substance stimulating the growth of yeast.

Streptococcus is more difficult to study because apparently it needs at least two substances for growth.

Although vitamine D has been obtained free from vitamine B, as far as our animal experiments have shown, the reverse is not true. It is evident, therefore, that most animal tests conducted up to the present were carried out with a mixture of vitamins B and D and will consequently have to be repeated as soon as a clear separation of the two substances can be effected. It may develop that the vitamine D, obtained from yeast, and the vitamine-like substance obtained from proteins, such as casein, may have some special function in the body, and such experiments are now being planned.

Regarding the possible identity of the substance promoting the growth of yeast with that influencing the growth of streptococcus, our present data are insufficient to venture a definite statement.

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# THE EFFECT OF SUBCUTANEOUS INJECTIONS OF SOLUTIONS OF POTASSIUM CYANIDE ON THE CATALASE CONTENT OF THE BLOOD.

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(Received for publication, June 20, 1921.)

In 1889 Geppert<sup>1</sup> concluded on the basis of an extensive series of experiments that potassium cyanide acted on the living organism through the mechanism of making the cells lose their power of oxygen utilization. He states that the picture is one of internal suffocation in the presence of excess oxygen. His experimental results showed that the oxygen consumption was very markedly diminished, and also that the carbon dioxide formed was very markedly diminished. Scientific investigators seem to have accepted Geppert's results and interpretation as final, since no experiments have been carried out since that time along precisely the same lines.

In recent years a theory has been built up to the effect that catalase of the blood (by which we understand the catalytic activity which greatly accelerates the breaking down of hydrogen peroxide) follows the oxidative capacity. Further, that anything which affects the catalase content of the blood must necessarily have a similar effect on the oxidative process in the organism. It appeared to us that potassium cyanide would be a satisfactory substance to apply, in testing out this theory.

The methods that had been used for the estimation of catalase at the time this work was planned, appeared to be rather crude, and so an apparatus was built in which the liberated gas could be measured under conditions corrected for pressure and where the shaking could be done mechanically under constant conditions.

<sup>1</sup> Geppert, J., *Z. klin. Med.*, 1889, xv, 307.

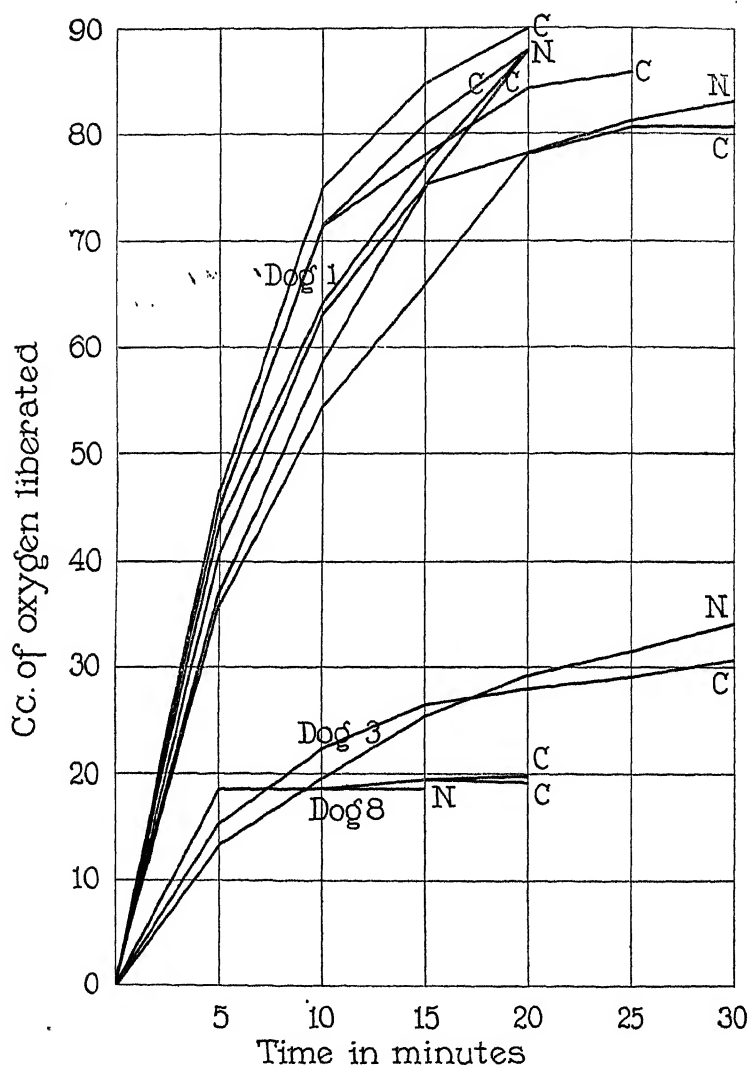


FIG. 1.

A description<sup>2</sup> of this apparatus is being published in another journal. Our determinations were carried out in triplicate and practically all the results given are the averages of closely agreed triplicates. In all but one of our series of experiments, samples of 0.5 cc. of oxalated blood were used. The exception was in the case of Dog 8 in Fig. 1, in which samples of 0.25 cc. were used. The sample was placed into a small aluminium vessel and floated on top of 75 cc. of equal volumes of hydrogen peroxide and distilled water. The stoppers were then carefully placed into the bottles, the gas levels adjusted, the stop-cocks on the bottles closed, and the machine was started. The volumes of gas, liberated, were read at 5 minute intervals, run off by a timer, and the shaking was continued until the curves became practically flat. From an examination of the curves obtained from normal bloods (Fig. 2) it is apparent that final readings taken at the end of a 10 minute period do not give results that can be satisfactorily compared. This 10 minute period has been used by a number of investigators in the estimation of catalase. These curves also show that there is quite a marked variation between different normal bloods. In these experiments potassium cyanide was injected subcutaneously in such amounts as to produce death. The blood samples were removed from the femoral artery by means of a cannula. A small quantity of powdered potassium oxalate was used to prevent clotting. The starred curve for Dog 4 in Fig. 3 was obtained from blood mixed with potassium cyanide *in vitro* in the same proportion as was injected. From an examination of the curves from our eight experiments (Figs. 1, 3, 4) it becomes apparent that there is a diminution in the catalase content of the blood in only one case. In these figures the curves marked N were obtained from normal blood and those marked C from blood after injection of potassium cyanide. This is true even when the blood was taken during coma, and in two cases where the blood was tested after death.

From the experimental results we conclude that lethal doses of potassium cyanide injected subcutaneously have practically no effect on the catalase content of the blood; secondly, that if the generally accepted theory of Geppert as to the mode of action of

<sup>2</sup> Welker, W. H., *J. Lab. and Clin. Med.*, 1921, vii, in press.

potassium cyanide is correct, there can be no connection between oxidase and catalase.

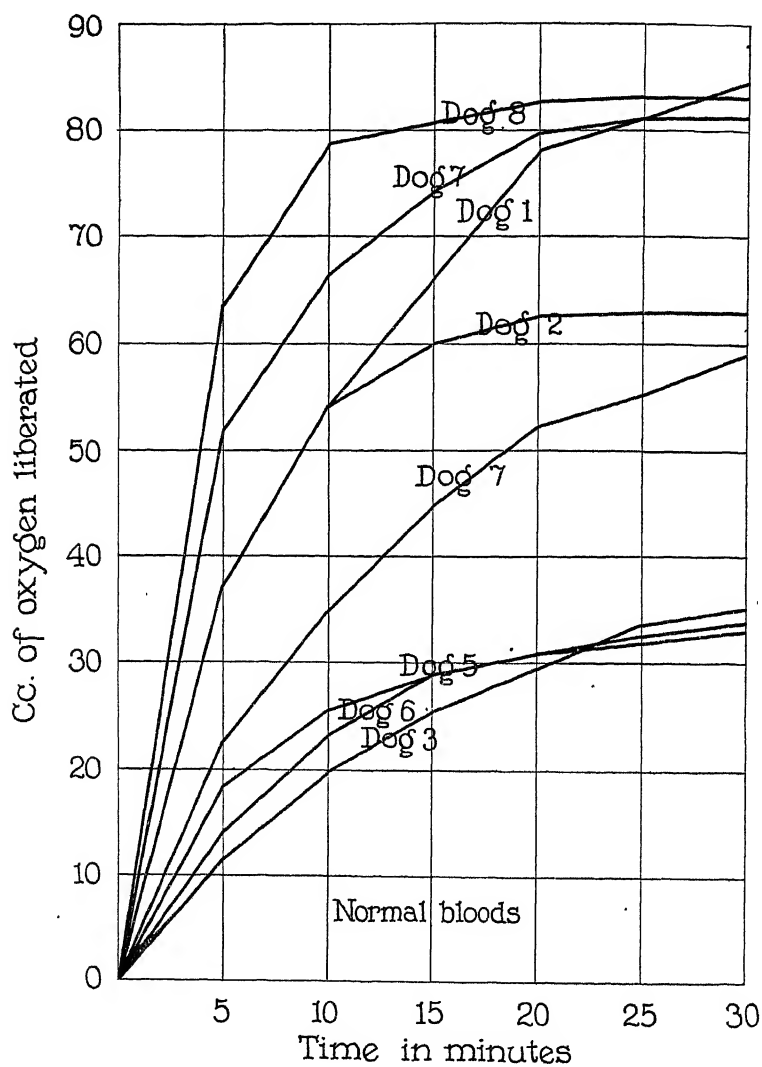


FIG. 2.

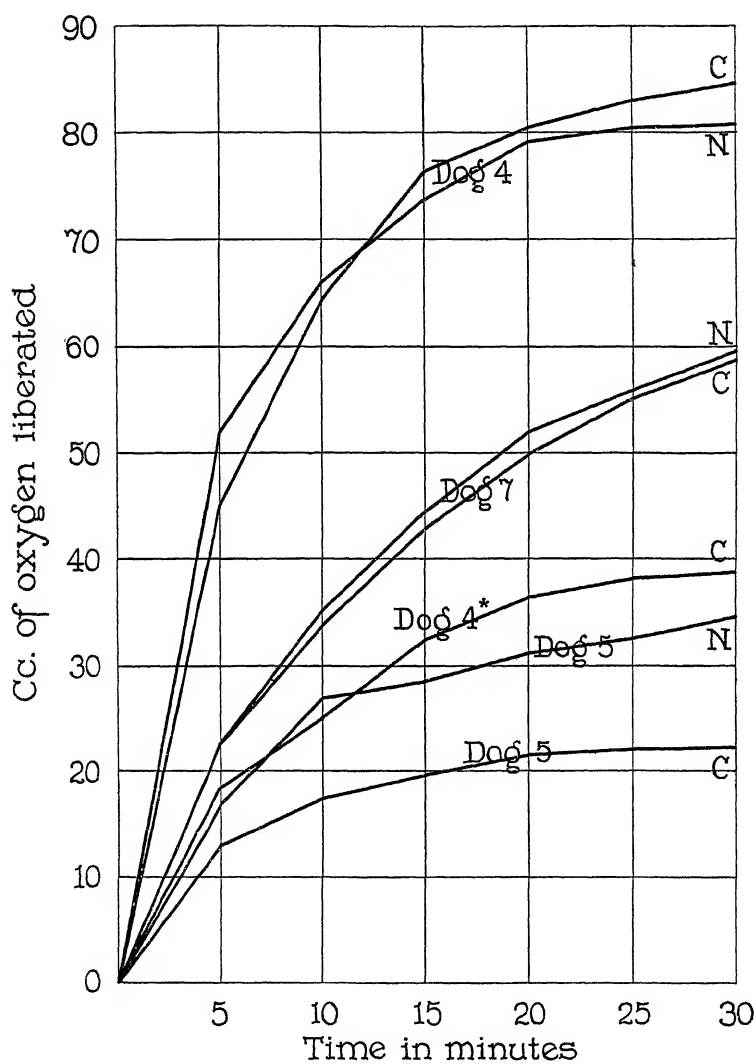


FIG. 3.

*Protocols.*

*Dog 1.*—Two samples of normal blood were taken at an interval of 15 minutes. 3 mg. per kilo of 5 per cent KCN solution injected at 11.50 a.m. Second injection of same dose at 12.35 p.m. Blood sample taken at 12.40 p.m. 6 mg. per kilo of 5 per cent KCN solution injected at 12.55 p.m.

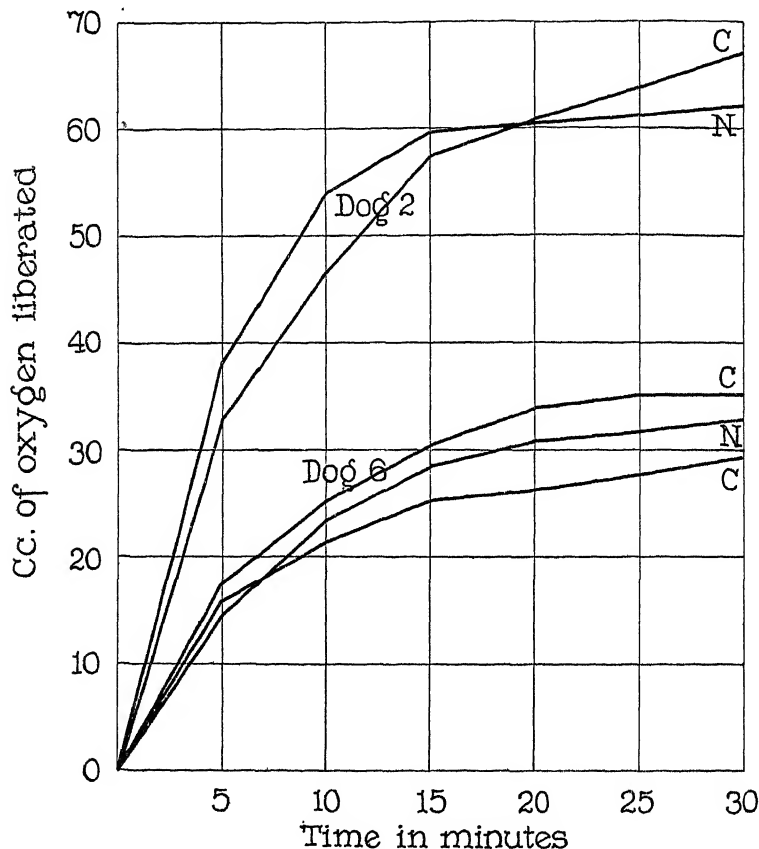


FIG. 4.

Blood sample taken at 1.05 p.m. Blood sample taken at 1.55 p.m. The animal was in convulsions at this point. Death occurred at 2.00 p.m. Another sample of blood was taken at this time.

*Dog 2.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 1.20 p.m. Injection repeated at 1.30 p.m. Death occurred at 1.45 p.m. Blood removed from the heart at 1.48 p.m.

*Dog 3.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 12.35 p.m. Death occurred at 1.00 p.m. Sample of blood removed from the heart within 30 seconds after the occurrence of death.

*Dog 4.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 2.15 p.m. Injection repeated at 2.30 p.m. Blood sample taken at 2.40 p.m. Death occurred at 2.50 p.m.

*Dog 5.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 11.40 a.m. Convulsions at 11.43 a.m. Blood taken at 12 noon. Death occurred at 12.20 p.m.

*Dog 6.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 11.11 a.m. Convulsions and labored breathing at 11.25 a.m. Blood sample removed at 11.38 a.m. and 1.55 p.m. Death occurred at 2.20 p.m.

*Dog 7.*—Blood taken for normal sample. 8 mg. per kilo of 5 per cent KCN solution injected at 10.15 a.m. Blood removed at 11.05 a.m. The excitement stage had reached its maximum at this point. Death occurred at 2.00 p.m.

*Dog 8.*—Blood taken for normal sample. 10 mg. per kilo of 5 per cent KCN solution injected at 4.05 p.m. 6 mg. per kilo of 5 per cent KCN solution injected at 4.30 p.m. Marked convulsions at 4.37 p.m. Blood sample taken at the point of death at 4.45 p.m.





## CITRIC ACID CONTENT OF MILK AND MILK PRODUCTS.

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Citric acid has been recognized as one of the normal constituents of milk for many years, but there is still some disagreement as to the forms in which it exists in this product. Soldner (1) claims the presence of potassium, magnesium, and calcium citrates, whereas Van Slyke and Bosworth (2) state that only sodium and potassium salts of this acid are present. Regardless of the exact form in which this constituent is found in milk, quantitative determinations have shown that there is normally between 0.1 and 0.2 per cent citric acid combined in one form or another.

Interest has been directed to the parallelism between the citric acid content of fruit juices and their antiscorbutic properties, and to the similar association of these factors in milk. If only natural products, unheated and without subjection to processing for preservation, were considered, the existence of a definite relationship between the two factors would be more acceptable. Reliable experimental evidence, however, shows that the antiscorbutic properties of natural foods are destroyed by heat whereas the citric acid content of the same products is not quantitatively affected by the application of heat even in excess of that required for the destruction of the antiscorbutic property. Sommer and Hart (3) have shown that milk may be autoclaved at 15 pounds pressure for 1 hour without causing a diminution in its citric acid content. They have also shown that the citrates of milk are not rendered insoluble by autoclaving for 20 minutes at 15 pounds pressure.

Since comparatively recent investigations by Hess (4, 5), Hart (6, 7), and Dutcher (8) and their coworkers have made available additional data on the antiscorbutic potency of milk as affected

by the feed of the lactating animal, and by what appears to be the method of processing in the preparation of various concentrated milk products, it has been considered desirable to record the variations in citric acid content of milk from individual cows while on a normal winter ration and when on a normal summer or pasture ration; also to determine the citric acid content of some well known concentrated milk products.

#### EXPERIMENTAL.

##### *Estimation of Citric Acid in Milk.*

Due to the small amount of citric acid present in milk and because of the possible variations which it was desirable to detect, it has been deemed advisable to incorporate details of the well known methods used in this series of analyses.

*Determination of Citric Acid in Milk.*—50 cc. of milk are treated with 10 cc. of dilute sulfuric acid (1:1) and thoroughly agitated. 2 cc. of 40 per cent potassium bromide solution and 20 cc. of a solution of phosphotungstic acid are then added. After a thorough mixing, the precipitate is separated by filtration. To the perfectly clear filtrate in an Erlenmeyer flask is added an excess of freshly prepared saturated bromine water (usually between 5 and 10 cc.). The mixture is then placed on the water bath at a temperature of from 48–50°C. for about 5 minutes. After removing from the bath, add rapidly from a burette 25 cc. of potassium permanganate solution (5 per cent) drop by drop with frequent interruptions, and with constant and vigorous shaking, avoiding a temperature during the oxidation exceeding 55°C. Set the flask aside until the hydrated peroxide of manganese begins to settle. The supernatant liquid should be dark brown showing an excess of permanganate. Add more permanganate if an excess is not indicated. When the precipitation assumes a yellow color and most of it is dissolved, add drop by drop a clear solution of ferrous sulfate until the hydrated peroxide of manganese and excess of bromine are removed. Allow the solution to cool, shaking occasionally. Allow the mixture to stand over night. Collect by means of gentle suction on a tared Gooch crucible provided with a thin pad of asbestos previously dried over sulfuric in a vacuum desiccator; wash with water slightly acidified with sulfuric acid and finally wash twice with water. Dry the precipitate to constant weight over sulfuric acid in a vacuum desiccator protecting the precipitate from strong light. The weight of the precipitate multiplied by the factor 0.424 gives the equivalent weight of anhydrous citric acid in the sample.

*Determination of Citric Acid in Milk Powder.*—Weigh 5 gm. of powder into a beaker and reconstitute with 45 cc. of warm water. Mix thoroughly and proceed as with liquid milk.

*Determination of Citric Acid in Sweetened Condensed Milk.*—Weigh out 25 gm. of the sample and add 200 cc. of 95 per cent alcohol. Mix thoroughly and filter. To the filtrate add enough 0.25 N barium hydroxide to almost neutralize the solution and then 5 cc. of 50 per cent barium acetate in order to insure an excess of barium. Add about 150 cc. of 95 per cent alcohol and reflux until the precipitate settles readily after being shaken. Filter and thoroughly wash the precipitate in the flask and on the paper with 95 per cent alcohol. Transfer the precipitate from the filter to the flask with a jet of hot water. Boil until alcohol can no longer be detected by odor and add enough sulfuric acid (1:5) to precipitate all of the barium originally present and to allow 2 cc. in excess. Evaporate to a volume of 60 or 70 cc.; cool and add an excess of bromine water. Filter and add 10 cc. of potassium bromide, then place on the water bath at a temperature of 48–50° C. and proceed as with liquid milk.

TABLE I.  
*Percentage of Citric Acid Recovered from Milk Products.*

	Liquid milk.		Liquid milk and sugar.		Evaporated milk.		Condensed milk.	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Original.....	0.132	0.129	0.131	0.130	0.202	0.204	0.096	0.090
After adding 0.02 per cent ..							0.110	0.104
“ “ 0.05 “ “	0.179	0.180	0.182	0.179	0.252	0.253	0.143	0.148
“ “ 0.10 “ “							0.190	0.197
“ “ 0.15 “ “	0.279	0.279	0.279	0.275				

The relative accuracy of these methods is shown in Table I in which is given the results of duplicate determinations on liquid milk with and without sugar, on evaporated milk, and on sweetened condensed milk; also duplicate results from each of these products after known amounts of citric acid in the form of sodium citrate had been added. It will be noted that the maximum variation in duplicate results does not exceed 0.006 per cent; it is believed therefore, that any significant variations occurring in the products examined were easily detected by the methods used.

*Citric Acid in Milk as Affected by Feed.*

In view of the work reported by Hess, Unger, and Supplee (4) in which it was shown that the milk produced from a highly concentrated ration contained less citric acid than that produced during pasture feeding, it has seemed desirable to obtain further

data on the amount of this constituent found in the milk of the same herd while receiving a normal winter ration and again during pasture feeding. Accordingly, samples were analyzed late in February and again late in June; the results are shown in Table II.

From the results in Table II it is evident that there is a wide variation shown in the milk from individual animals receiving the same feed as represented by the difference between 0.121 and 0.182 per cent (Herd I). While the evidence pointing toward a variation

TABLE II.

*Citric Acid Content of Milk from Winter Ration and from Pasture Feeding.*

Herd.	Cow.	Winter ration.		Summer pasture.	
		Citric acid.	Feed.	Citric acid	Feed.
		<i>per cent</i>		<i>per cent</i>	
I	1	0.173	Hay, distiller's grains, ensilage, corn stover, molasses.	0.174	Fresh grass only.
	2	0.121		0.114	" " "
	3	0.182		0.156	" " "
II	1	0.145	Hay straw, cottonseed, meal.		" " "
	2	0.155		0.148	" " "
	3	0.106		0.130	" " "
III	1	0.139	Hay, oil meal, corn-meal, bran.	0.164	" " "
	2	0.119		0.138	" " "
	3	0.139		0.160	" " "

in citric acid content as affected by the different feeds is not conclusive, there is, nevertheless, a tendency toward a higher percentage of this constituent in the winter milk of cows receiving ensilage and corn stover than in the milk of those herds receiving only hay as roughage. When summer and winter milks from each herd are compared there is a significant difference only in the case of Herd III in which the milk from pastured cows contains a uniformly higher citric acid content. The average citric acid content of the milk from all cows on a winter ration was 0.142 per cent and from all cows when on pasture was 0.148 per cent.

*Citric Acid Content of Concentrated Milk Products.*

Since one of the purposes of this paper is to furnish analytical data showing the citric acid content of concentrated milk products, it is desirable to briefly mention that in the manufacture of condensed, evaporated, and desiccated milks heat is applied in amounts varying from 110–112°C. for a few seconds in the manufacture of powdered milk by the Just process, to sterilization under steam pressure in the case of evaporated milk. Therefore, the results from the products analyzed will adequately cover the temperature range to which concentrated milk products are subjected during process of manufacture.

TABLE III.

*Citric Acid Content of Condensed and Evaporated Milks.*

Sample.	Milk.	Citric acid in product.	Citric acid calculated to liquid milk basis.
		<i>per cent</i>	<i>per cent</i>
1	Evaporated.....	0.168	0.084
2	“ .....	0.302	0.151
3	“ .....	0.295	0.147
4	“ .....	0.211	0.105
5	“ .....	0.255	0.127
6	“ .....	0.203	0.101
7	Sweetened condensed .....	0.094	0.078
8	“ “ .....	0.124	0.103

The citric acid content of six different brands of evaporated milk and two brands of sweetened condensed milk are shown in Table III; also included in this table is the citric acid content calculated to the original liquid milk basis assuming a concentration ratio of 2 to 1.

The citric acid content of milk powder made by the spray process is shown in Table IV. The concentration ratio used for calculating to the liquid milk basis is 1 to 8.5 and 1 to 12 respectively for whole milk and skimmed milk.

It has been possible to check up very closely on the citric acid content of powder made by the Just process by determining the citric acid and total solids before drying, for comparison with the dried product and with the reconstituted milk correctly diluted

on the basis of the data obtained from the total solids determinations. The results of these determinations are shown in Table V.

The results from the different concentrated and desiccated milks do not show any variation in citric acid content which could

TABLE IV.  
*Citric Acid Content of Milk Powder (Spray Process).*

Sample	Milk.	Citric acid in product.	Citric acid calculated to liquid milk basis.
		<i>per cent</i>	<i>per cent</i>
1	Whole milk powder.....	1.26	0.148
2	" " " .....	1.22	0.143
3	" " " .....	1.23	0.144
4	Skimmed milk powder.....	1.70	0.141
5	" " " .....	1.50	0.125
6	" " " .....	1.45	0.121

TABLE V.  
*Citric Acid Content of Milk Powder (Just Process).*

Sample.	Milk.	Citric acid in milk before drying.	Citric acid in powder.	Citric acid in accu- rately re- constituted powder.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Skimmed milk.....	0.139	1.37	0.135
2	" " .....	0.105	1.05	0.104
3	" " .....	0.165	1.90	0.166
4	Part skimmed milk.....	0.138	1.29	0.138
5	" " " .....	0.110	1.05	0.110
6	" " " .....	0.170	1.76	0.167
7	Whole milk.....	0.141	1.15	0.141
8	" " .....	0.103	0.776	0.102
9	" " .....	0.173	1.45	0.177

not possibly be explained by variations in the liquid milk prior to manufacture. In the case of the Just process powders where complete data were available for reconstituting powder to the original milk basis, the citric acid content before and after drying is in full agreement. These results are therefore in full accord

with those of Sommer and Hart (3) and others in that the heating of milk does not cause a decrease in its citric acid content. The results also serve as further evidence to the lack of a definite relationship between citric acid content and antiscorbutic potency, as there is experimental evidence showing that some of these concentrated milk products apparently lack this vitamine, whereas in others it is retained to a high degree.

*Citric Acid Content of Milk as Affected by Age and Bacterial Quality.*

From the preceding data it has appeared that the variations in citric acid content of concentrated milk products could be explained

TABLE VI.  
*Citric Acid Content of Milk as Affected by Aging.*

Sample.	Age.	Acidity.	Citric acid.
		<i>per cent</i>	<i>per cent</i>
Raw.....	Fresh.	0.182	0.134
" .....	3 days.	0.250	0.129
" .....	4 "	0.500	0.121
" .....	14 "	0.550	0.104
" .....	28 "	0.650	0.042
Pasteurized.....	Fresh.	0.160	0.134
" .....	7 days.	0.160	0.134
" .....	14 "	0.180	0.134
" .....	28 "	0.550	0.087

by the variations in the milk from individual animals rather than by the method of manufacture. Another possible cause of variation is suggested, however, by the observations of Kunz (9) to the effect that citric acid gradually decreases with the aging of milk, particularly when soured. The utilization of citrates by certain microorganisms is an established fact and it would seem quite possible that under certain conditions and in the presence of certain species of bacteria, the citric acid content of milk might be appreciably lowered. In order to secure information on this point, a sample of milk held raw and after pasteurization for a period of 28 days was analyzed for developed acidity and citric acid content at regular intervals. The samples were held at low



temperatures after the first 3 days. The results of these determinations are shown in Table VI.

Even though the results given in Table VI show a marked decrease in citric acid content due to aging, there is considerable doubt as to just how far these results can be used as an explanation of the variations recorded in the preceding tables, particularly in view of the knowledge that milk for manufacturing purposes must of necessity be fresher and of better quality than that in which the diminution in citric acid was detected. The results, however, are significant as indicating the general tendency toward reduction in citric acid content in milk of poor quality.

#### SUMMARY.

The conclusions which may be drawn from these investigations follow.

There is a marked variation in citric acid content of the milk from individual animals, which may be explained on the basis of the data shown herein as due to the individuality of the particular animal. Certain data, however, indicate that the ration may have a slight effect upon this constituent.

There is apparently no effect upon the citric acid content of milk caused by heating during the manufacture of evaporated, condensed, and dried milks. The results indicate that the amount found in each of these products, if subject to variation, must be attributed to causes other than heat.

The parallelism between citric acid content and antiscorbutic properties does not hold true in the case of concentrated milk products; the potency of this factor has been shown to be absent in some of the heated products and present in others. The citric acid content however, seems to be present in all of them apparently to the same degree as found in natural raw milk.

The citric acid content of milk decreases during aging in the presence of high developed acidity, and is more rapid in raw milk than in pasteurized milk. It is quite probable that the effect of acidity and age is not applicable in causing as marked a diminution of citric acid in milk used for manufactured products as is shown by the data presented herein.

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# THE AMMONIA CONTENT OF THE BLOOD, AND ITS BEARING ON THE MECHANISM OF ACID NEUTRALIZATION IN THE ANIMAL ORGANISM.

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A review of the very full literature upon the concentration of ammonia in blood discloses much confusion and divergence in the reported values. For example, the range of values for the systemic blood of normal dogs is from a few hundredths of a mg. (Folin and Denis, 1) to 5 mg. (MacCallum and Voegtlin, 2) or more of ammonia nitrogen in 100 cc. Even more pronounced are variations in the data of comparable pathological states; yet in many instances the blood ammonia has been determined purely incidental to the investigation of problems of intermediary metabolism, and made the basis for hypotheses whose validity depends upon not only the relative but absolute accuracy of the analyses. With improvements in technique, and a growing appreciation of the inherent difficulties in the estimation of ammonia in blood there has been a preponderant (but by no means uniform) trend to the lower extreme of values, until, according to Myers (3), "there appears to be some question at the present time as to whether ammonia actually exists in the blood."

In 1896, Nencki and Zaleski (4) adapted to the estimation of ammonia in blood the vacuum distillation procedure originally proposed by Boussingault (5) and more recently employed by Wurster (6). 50 to 100 gm. of blood and an equal volume of filtered lime water were distilled for 3 hours, at a pressure of 10 to 15 mm. and temperature not exceeding 35°C. The ammonia was received in 0.1 N sulfuric acid and the excess acid titrated by 0.025 N sodium hydroxide solution, with methyl orange indicator. By this method Nencki and Zaleski found in 100 gm. of dog blood the following values: arterial blood, from 1.4 to 2.8 mg. of ammonia; portal blood, 5.2 mg.; and hepatic venous blood, 1.9 mg. They realized, however, that

higher temperatures, longer distillation, or greater concentration of alkali, would give higher values.

This method found immediate application in the very interesting series of experiments which were then being carried out by the Nencki-Pawlow school on dogs with Eck fistula, with the view of determining the site of urea formation in the animal body. Various experimental evidence had accumulated indicating that urea formation was a function of the liver. Thus, Minkowski (7) had shown that after extirpation of the liver in geese there is a very great fall in uric acid excretion and a corresponding increase in ammonia excretion. Von Schröder (8) had disposed of the old notion that the kidneys were able to form urea, and had found that perfusion of the liver with blood containing ammonium carbonate produced an increase in the urea content of the blood. Furthermore, it had been observed that Eck fistula dogs show symptoms of acute poisoning on a heavy meat diet. Nencki, Pawlow, and Zaleski (9), Nencki and Pawlow (10), Salaskin (11), Salaskin and Zaleski (12), and others, employing the new vacuum distillation method, found that the blood of the portal system contains three to four times as much ammonia as arterial blood; that after Eck fistula operation in dogs, with extirpation of the liver or ligation of the hepatic arteries, the ammonia content of arterial blood and the excretion of ammonia are increased; that during the acute poisoning of meat-fed Eck fistula dogs the ammonia content of the arterial blood approaches the values found for portal blood. The conclusion seemed well established that the chief site of urea formation, in mammals, at least, is the liver, and that the ammonia split off from protein during intestinal digestion is converted into urea in the liver.

The experimental findings and conclusions of the Petersburg school did not go unchallenged even at this early date. Biedl and Winterberg (13) were unable to find a constant difference between the ammonia of arterial and portal blood, and stated that the ammonia value is directly dependent, within wide limits, on the ratio between the amounts of blood and lime water employed in the Nencki-Zaleski procedure. Nencki and Zaleski (14) confirmed this criticism of their original method and introduced several modifications including the use of magnesium oxide instead of lime water, and distillation at a lower temperature and for a shorter time. With the method thus modified they found as the average value for arterial blood of fifteen dogs 0.35 mg. of ammonia, and for portal blood 1.45 mg. of ammonia, in 100 gm. of blood. They also observed that where tissues and blood stood for 24 hours, even on ice, higher values for ammonia were obtained.

In view of the demonstrated inaccuracy of the analytical method upon which the conclusions of the Petersburg school were based, Horodyński, Salaskin, and Zaleski (15) considered it necessary to repeat experiments designed particularly to settle the essential question of the excess ammonia of the portal blood. They certified the absolute accuracy of the modified Nencki-Zaleski method, and reported the following average for ammonia (mg. of ammonia in 100 gm. of blood): normal dogs, arterial

blood, 0.41; fasting dogs, arterial blood, 0.42; normal dogs, portal blood, 1.85; fasting dogs, portal blood, 1.29.

In 1902, Folin (16) revived Boussingault's (5) idea of quantitatively removing ammonia from solutions by aeration and adapted it to the estimation of ammonia in urine and other animal fluids. An important detail of Folin's new method was the use of sodium carbonate and sodium chloride to liberate the ammonia, since he found that blood is rich in labile organic compounds from which even weak alkalies, as calcium or magnesium hydroxide, can split off ammonia by hydrolysis at room temperature. Folin employed 50 cc. of blood which were kept cold by ice during the 5 hour aeration, and estimated the ammonia by titration of the excess acid into which it was aerated. He found in the arterial blood of dogs 0.5 to 0.6 mg. of ammonia per 100 gm.

The aeration method did not at once (nor has it to this day) displaced the vacuum distillation technique. Beccari (17), employing the latter procedure, and estimating the ammonia in the distillate as the chloroplatinate, obtained on three dogs the average value of 0.8 mg. of ammonia in 100 gm. of blood.

Voegtlin and King (18), without giving experimental data, say: "The large output of ammonium salts in the urine, as well as high ammonia content of the blood, in clinical conditions involving acidosis, suggested that these salts may play an important rôle in producing the symptoms of these diseases."

Wolf and Marriott (19), using protein-free blood filtrates and a vacuum distillation-titration procedure, found from 1.4 to 4.9 mg. of ammonia nitrogen in 100 cc. of fresh ox blood. Employing Folin's aeration-titration technique, Carlson and Jacobson (20) obtained on normal cats an average of 1.57 mg. of ammonia per 100 cc. of blood, and on normal foxes, 2.388 mg.; on thyroparathyroidectomized cats and foxes, 2.533 and 3.561 mg., respectively. With this same method, Greenwald (21) was unable to obtain satisfactory duplicates, but concluded from his experiments that there is no increase in the blood ammonia of dogs during tetany following parathyroidectomy. Carlson and Jacobson (22), estimating the ammonia by Nessler's reagent instead of titration, following aeration, obtained much smaller values for the blood ammonia than they had previously found and were unable to find any marked or constant increase after parathyroidectomy. Hopkins and Denis (23), by aerating and titrating, found in normal blood from 0.6 to 3.2 mg. of ammonia per 100 cc.

Medwedew (24) made use of a modified vacuum distillation-titration process in which sodium carbonate was employed to liberate the ammonia from the blood. He found that blood, oxalated and preserved under aseptic conditions for 24 hours, shows a significant increase in its ammonia content. When the analysis was undertaken within 10 to 12 minutes after collection he found as the average value in mg. per 100 gm. of blood: in normal dogs, 0.56; in thyroparathyroidectomized dogs, 0.79; and in long fasted dogs, 1.81. The most interesting part of Medwedew's work is his discussion of the changes in the blood ammonia on standing under aseptic

conditions. Thus, in the blood of normal dogs there is an initial slow increase which becomes accelerated in the manner of an autocatalyzed reaction. In the blood of thyroparathyroidectomized dogs there is a much more rapid increase beginning immediately after collection; but in either case, at the end of 24 hours the ammonia has become constant and remains stationary at about the same level. In the blood of long fasted dogs, on the other hand, there is a slow, progressive decrease during the first 6 to 8 hours, after which a slow, progressive increase sets in and continues for 20 to 25 hours but never quite reaches the original value. Medwedew concluded that in blood there is an equilibrium between ammonia and a certain quantity of substance or substances which decompose with the liberation of ammonia. These reactions are catalyzed by a deamidase and a synthetic enzyme of the plasma, while the cells also contain a deamidase. In normal blood the two antagonistic enzymes of the plasma are in equilibrium, but after collection there is a diffusion of the cell deamidase. In the blood of thyroparathyroidectomized dogs the synthetic enzyme of the plasma is diminished in concentration or repressed. While in the blood of fasted dogs the synthetic enzyme at first predominates, but is finally balanced by the diffusion of the cell deamidase. Medwedew believed that these various relations are so constant that they may be formulated mathematically.

In 1912, Folin and Denis (1) published a notable contribution to the subject of ammonia in blood, and reported values far lower than any previously found. The essential details of their analytical method were: the use of small amounts, 5 to 10 cc. of blood; rapid and short aeration, 20 to 30 minutes; estimation of the ammonia by use of Nessler's reagent instead of by titration. In the carotid blood of cats they found from 0.03 to 0.08 mg. of ammonia nitrogen per 100 cc. Further they found that the ammonia of the mesenteric vein of the small intestine is not materially greater, and may be less, than in the portal vein; whereas the ammonia of the mesenteric vein of the large intestine is invariably greater than that in the portal vein. Folin and Denis therefore came to the conclusion that the large intestine (because of the bacterial action therein) is the chief or most constant source of the ammonia found in portal blood, and state: ". . . the portal ammonia is hereby largely robbed of the peculiar interest which has attached to it for the past fifteen years, and since the amount of ammonia in other blood is almost infinitesimal under ordinary normal conditions this too becomes a rather unimportant feature of normal metabolism." They point out that: "The blood decomposes spontaneously (and particularly in the presence of alkalis capable of setting free the ammonia) at all temperatures even when kept on ice. The ammonia thus produced by decomposition in the course of a few hours is much greater than the preformed ammonia present in the strictly fresh blood. . . ."

The values found by Folin and Denis have not gone unchallenged. Matthews and Miller (25) found with the same technique 0.35 mg. of ammonia per 100 gm. of arterial dog blood. Denis (26) reported from 1.0 to 5.5 mg.

of ammonia nitrogen in 100 gm. of blood from various species of fish, but states that further (unpublished) observations on the ammonia content of normal and pathological human blood have shown that ammonia is present to the extent of only a fraction of a mg. per 100 gm. of blood. Jacobson (27) used larger quantities of blood and aerated for 4 hours, estimating the ammonia by Nesslerization, and obtained values ten to twenty times as large as those of Folin and Denis. Rohde (28) carried out vividiffusion experiments on dogs and determined the ammonia in the dialysate when equilibrium had been reached between the dialysate and the blood. In one experiment the dialysate contained 0.18 mg. of ammonia nitrogen per 100 cc.; in a second experiment, 0.30 mg. Rohde also made direct analyses of dog's blood drawn with aseptic precautions, employing Folin's original aeration-titration procedure, aerating for 3 hours, with the addition of saturated sodium carbonate solution to liberate the ammonia. When aeration was begun immediately and carried out at room temperature the value obtained was 0.72 mg. per 100 cc. of blood; when the aeration cylinder was placed in ice the value fell to 0.28 mg. The same blood after having stood for 24 hours on ice, with chloroform and toluene (at the end of which time bacteriological examination gave negative cultures), gave 1.78 mg. of ammonia nitrogen per 100 cc. when aerated at room temperature, and 0.44 mg. when the aeration cylinder stood in ice water during the analysis. Since Rohde found no increase in the ammonia content of the dialysate even on prolonged standing, she concluded that blood contains labile substances which easily split off ammonia (thus confirming Folin, and Medwedew), and that these are to be sought in the non-dialyzable constituents of the blood. Gettler and Baker (29) found, by the Folin-Denis method, from 0.4 to 1.1 mg. of ammonia nitrogen in 100 cc. of normal human blood, with an average for 30 cases of 0.51 mg. Bang (30) aerated protein-free blood filtrates and determined the ammonia by titration. In the blood of normal rabbits he found 0.81 to 1.27 mg. of ammonia nitrogen per 100 gm. He says of these values: "...sind sie bedeutend höher als Folin's Normalwerte, die jedoch unmöglich richtig sein können." Bang found in the blood of a rabbit which showed symptoms of acute poisoning, following the ingestion of 10 gm. of urea 2 hours before, 6.25 mg. of ammonia nitrogen per 100 gm. In other rabbits which had received lethal doses of ammonium carbonate, Bang found from 3.8 to 8 mg. of ammonia nitrogen per 100 gm. of blood. Henriques and Christiansen (31) pointed out the doubtful validity of all experimental data and deductions therefrom, including those of Medwedew, which depend upon vacuum distillation-titration analyses. Furthermore, "Folin und Denis' sehr niedrige Werte (0,03 mg. oder 'Spuren') müssen als irrtümlich betrachtet werden." The analytical procedure employed by Henriques and Christiansen was as follows: 20 cc. of the freshly drawn blood are diluted at once with 4 volumes of ethyl alcohol (to prevent foaming and bacterial action), sodium carbonate is added, and the mixture aerated for 3 hours into dilute sulfuric acid. The content of the receiving flask is then made up to 80 cc. with distilled water and the alcohol distilled off. Sodium hydroxide is



next added and the ammonia distilled through a silver tube condenser into 5 cc. of 0.005 *N* sulfuric acid. The excess acid is titrated indirectly by *N*/280 sodium thiosulfate (1 cc. = 0.05 mg. of *N*) using potassium iodate and starch indicator. In arterial blood they found from 0.15 to 0.38 mg. of ammonia nitrogen per 100 cc., and in portal blood from 0.25 to 0.91 mg. They observed little variation in the ammonia content of blood from different animals, and no difference between venous and arterial blood. The ammonia content of blood which stood for a short time was observed to increase, and aeration at temperatures slightly above 16° C. gave higher values than normal. The introduction of large quantities of urea or ammonium salts into the alimentary canal of rabbits did not give uniform results. In those rabbits which showed no symptoms of poisoning there was only a slight rise in the blood ammonia, whereas convulsions were always attended by a marked rise, in one instance reaching 7.38 mg. of ammonia nitrogen per 100 cc. of blood. Gad-Andersen (32) found that the concentrations of ammonia in muscle and blood are identical. His estimation of ammonia was made by a new micro method which was not described. Values for various muscles of from 0.3 to 0.8 mg. of ammonia nitrogen per 100 gm. of tissue were found. All earlier values for muscle, which were usually about twenty-five to thirty times higher than for blood, were due to a transformation of urea into ammonia after death. This transformation may be prevented by the method described. When muscle tissue was allowed to stand under conditions favorable to the transformation, the sum of the urea nitrogen and ammonia nitrogen remained constant, although the initial values of the two were reversed. Morgulis and Jahr (33) absorbed the ammonia from protein-free filtrates by permutit, from which the ammonia was subsequently liberated by sodium hydroxide, and Nesslerized. By adding known amounts of ammonia to the blood and to a control they were able to make the color comparison in an ordinary colorimeter. They found from 0.18 to 0.30 mg. of ammonia per 100 gm. of blood. Myers (34) aerated the ammonia from blood into a definite volume of 0.2 *N* sulfuric acid containing 0.05 mg. of ammonium sulfate per cc. This permitted Nesslerization and estimation in an ordinary colorimeter. Myers reported in the blood of various invertebrates from 0.00 to 1.05 mg. of ammonia nitrogen per 100 cc., with a general rough parallelism between the ammonia nitrogen and total non-protein nitrogen. In whale blood (which was obtained for analysis 3 to 4 hours or longer after death) Myers found 2.4 to 14.5 mg. of ammonia nitrogen per 100 cc. Myers corroborates Barnett's claim that there is a decided increase in the ammonia of blood on standing.

The only reported values of ammonia in blood of the same order as those found by Folin and Denis were obtained by Barnett (35) who devised an aeration-micro-titration procedure. Using capillary pipettes, methyl red indicator, and a sodium acetate-acetic acid mixture for a fixed comparative end-point, Barnett was able to obtain a sharp end-point with 0.005 cc. of 0.005 *N* alkali. He found in oxalated human blood, whose analysis was begun within 1 to 3 minutes after drawing, from 0.00 to 0.05 mg. of ammonia

per 100 cc. The same bloods 30 minutes later showed from 0.07 to 0.15 mg. of ammonia per 100 cc. Barnett stated that the results of analyses of ammonia in blood "are largely merely a measure of the extent to which the labile ammonia-yielding bodies of the blood have been disintegrated. . . . no means of overcoming this difficulty has been devised, and the best we can do is to minimize the error from the ammonia increase by beginning the aeration as soon as possible after the blood is drawn, and completing the analysis as rapidly as possible." Barnett and Addis (36), employing the above micro-titration technique, found in the blood of rabbits, following large doses of urea given by mouth, or directly into the bowel, or intravenously, marked increases in the ammonia content. In one instance the blood ammonia rose to 11 mg. in 100 cc.

If the extremely low values of blood ammonia reported by Folin and Denis, and Barnett, are accepted it becomes a very interesting problem to account for the relatively enormous quantities of ammonia excreted even in normal urine. A calculation will make clear the nature of the problem: If the rate of blood flow through the kidneys is 150 cc. per minute per 100 gm. of kidney tissue (37), and the total weight of kidney tissue is 300 gm., then the 24 hour volume of blood passing through the kidneys is 648 liters. Assuming that 100 cc. of arterial blood contains 0.1 mg. of ammonia nitrogen, and that this is *completely* removed, the total output of ammonia nitrogen in the urine for 24 hours would be 0.648 gm. This value is very close to that actually found for the normal, average individual, but the assumed figure for arterial blood is appreciably higher than that which we usually find, and it is scarcely conceivable that the kidney is 100 per cent efficient in the excretion of ammonia. Further, so low a concentration of blood ammonia is entirely inadequate to account for the high urinary ammonia found in various pathological conditions or after acid ingestion. It would seem, therefore, that one of the following possibilities must be true: *First*, the blood ammonia is higher than the value assumed above; *second*, ammonia is not present as such in the blood, but is in a loose combination readily split by the kidney although not easily dissociated under the conditions of analyses hitherto employed; or *third*, the kidney itself forms the ammonia which it eliminates. An investigation of these several hypotheses was the object of the work here reported.

## ANALYTICAL PROCEDURE.

Of the various procedures which have been proposed for the estimation of ammonia in blood the combination of aeration and Nesslerization seems least open to criticism. Salkowski (38) very early pointed out that milk of lime effects a decomposition of protein which invalidates ammonia determinations in protein-containing mixtures. Folin and Denis (1) state that "when distillation methods are applied, whether in the vacuum or otherwise, the determination becomes little less than a measure of the decomposition." Matthews and Miller (25) have well expressed the difficulties inherent in the estimation of small amounts of ammonia by titration: "It is well known that most indicators do not react with the greatest precision and therefore are not applicable when very small amounts are to be taken into account. This is especially true with ammonia, which is likely to form hydrolyzed salts with the indicator and thus give an indefinite end-point. In fact when not more than 0.5 mgm. of ammonia is absorbed in 100 cc. of  $\frac{N}{16}$   $H_2SO_4$  solution, it is impossible to titrate with sufficient accuracy to even approximate the actual amount, the limits of error being several times greater than the amount of ammonia to be determined . . . . Nessler's method . . . . is known to be accurate enough to distinguish 0.01 mgm. of ammonia and is therefore at least ten times more accurate than the titration methods heretofore ordinarily used. In fact it is the only known method accurate enough to determine quantities of ammonia ranging from 0.01 to 0.05 mgm." Another serious objection to the titration method is the difficulty of keeping standard solutions as dilute as those employed. This applies particularly in such micro-titration technique as that employed by Barnett (35), whose highest value for ammonia in human blood, 0.05 mg. of ammonia in 100 cc., corresponds to about 0.03 cc. of the 0.01 N hydrochloric acid used for the amount (10 cc.) of blood taken for analysis. The direct Nesslerization of protein-free blood filtrates, proposed by Morgulis and Jahr (33), whether or not permutit is used to absorb the ammonia, is open to Folin's (39) criticisms, and is not worthy of detailed consideration.

In connection with the determination of ammonia in blood by the aeration method it is well to bear in mind that this determination involves the measuring of smaller quantities of material than are dealt with in perhaps any other analytical process. Furthermore, the substance to be determined is present in the air of laboratories in appreciable quantity, and is contained in most reagents in detectable amounts. These considerations show that the determination of ammonia in blood can be of sufficient accuracy only when the most rigid precautions are observed at every stage of the process. Indeed it required several weeks of unrelenting effort before we were able to obtain distilled water blanks closely approximating the same ammonia-free water without aeration. Rubber stoppers may absorb ammonia in appreciable amounts from the laboratory air, which comes off slowly during the aeration. Triple acid washing of the air prior to its introduction into the blood is necessary for complete removal of the ammonia originally present.

The analytical procedure employed by us is essentially the Folin-Denis method with added precautions of technique which we believe are essential for correct results. The exact procedure is as follows: 5 cc. of oxalated<sup>1</sup> and well mixed blood is aerated for 10 minutes, the ammonia being received in 5 cc. of ammonia-free water<sup>1</sup> containing 2 to 3 drops of 0.1 N hydrochloric acid.<sup>1</sup>

<sup>1</sup> Reagents. Potassium oxalate used to prevent clotting was obtained ammonia-free by repeated recrystallization from ammonia-free water. Ammonia-free water was prepared by distilling dilute sulfuric acid, and collecting only a middle fraction. 0.1 N hydrochloric acid was prepared by diluting concentrated acid from a freshly opened bottle; 3 drops of the dilute acid in 5 cc. of ammonia-free water gave no trace of color with Nessler's reagent. The carbonate-oxalate solution employed was made by dissolving 8 gm. of anhydrous potassium carbonate and 12 gm. of crystalline potassium oxalate in distilled water, boiling down rapidly to half volume, diluting with ammonia-free water, and again boiling down to a small volume, and finally diluting to 80 cc. with ammonia-free water. The caprylic alcohol used was redistilled from a lot obtained from Eimer and Amend; we could detect no effect of the addition of 1 drop of the alcohol in blank determinations. The Nessler reagent was prepared according to the directions of Bock and Benedict. Standards containing 0.01, 0.02, 0.03, 0.04, 0.05, 0.075, 0.10, 0.15, 0.20 mg. of ammonia nitrogen per 100 cc. were readily prepared by dilution of a standard solution of ammonium chloride.

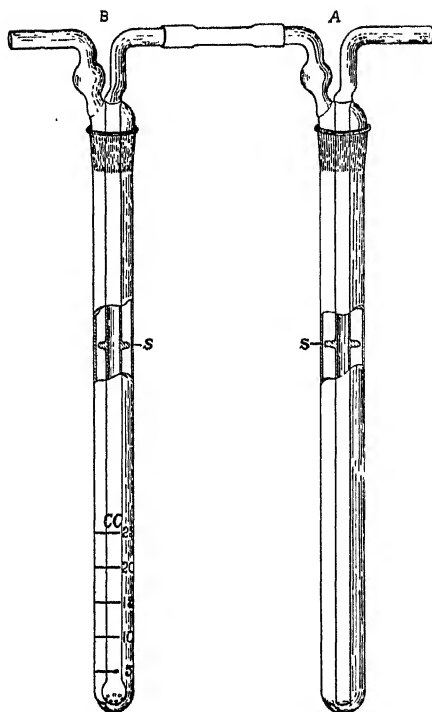


FIG. 1. The tubes are 300 mm. tall, exclusive of stoppers, and are made of good quality *white* glass. The internal diameter of Tube A is 20 mm., of Tube B 19 mm. The ground in stoppers carry the inlet and outlet tubes. (We have found it practically impossible to get absolute blanks when rubber-stoppered tubes are used.) Baffle plates, *S*, of glass are fused to the inlet tubes. These plates have a clearance of about 1 mm., and are very effective in preventing spray being carried over by the air current. They serve also to break the otherwise troublesome "foam rings" which slide up the tubes toward the end of the aeration when most of the caprylic alcohol has been driven out. The baffle plates, together with the height of the tubes, make possible a very rapid aeration. If desired, cotton or glass-wool may be placed in the bulbs of the outlet tubes as a further precaution against the carrying over of spray, but we have not found this necessary or desirable. The tubes are connected by rubber tubing, and any number of sets may be connected in series. This apparatus was made for us by Wm. T. Wiegand, 141 Lexington Ave., New York City.

To liberate the ammonia from the blood we use 1 cc. of a potassium carbonate-oxalate solution.<sup>1</sup> 1 drop<sup>2</sup> of caprylic alcohol<sup>1</sup> in the blood tube suffices during the short aeration to prevent frothing in both the blood tube and the acid collection tube. The special construction of the tubes (Fig. 1) allows an aeration rate of 4 liters per minute, which we obtain with a suction pump. The air is washed through a train of three<sup>3</sup> sulfuric acid absorption bottles to remove ammonia and then through a 1 per cent hydrochloric acid solution to saturate the air with moisture, before reaching the blood tube. When the aeration is completed the solution in the receiving tube is Nesslerized without transferring it,<sup>4</sup> 2 drops of undiluted Nessler reagent<sup>1</sup> being added. The ammonia is then estimated by transverse comparison with similarly and simultaneously Nesslerized standards<sup>1</sup> contained in tubes of the same internal diameter as that of the receiving aeration tube.<sup>5</sup>

The possibility of accurately recovering very small amounts of ammonia added to water by the procedure described was established by the results given in Table I.

Our first experiments with blood were designed to study the effect of standing on the ammonia content of the blood. No aseptic precautions were observed in drawing the blood, which was oxalated and kept in stoppered tubes on ice (except where otherwise noted) until used. Portions of the blood were successively aerated three times in a period of an hour. These samples, of course, stood in contact with the carbonate. Other portions

<sup>2</sup> More than 1 drop of caprylic alcohol may cause turbidity in the Nesslerized solution, particularly if the concentration of ammonia nitrogen is less than 0.05 mg. per 100 cc.

<sup>3</sup> Bock and Benedict (40) have shown that a single acid wash bottle does not completely remove the ammonia from air. This fact does not necessarily discount the use of a single absorption tube for the ammonia of blood, since the amount of ammonia escaping absorption appears to be a factor of the concentration of ammonia in the air.

<sup>4</sup> It is obvious that the inner absorption tube need not be washed down unless the "unknown" is to be diluted beyond its original volume.

<sup>5</sup> Differences of 0.01 mg. of ammonia nitrogen in 100 cc. are readily estimated in this manner, up to 0.20 mg. Higher concentrations than this are compared in an ordinary colorimeter. The standard tubes are graduated to permit dilution of the prepared standards when necessary to obtain intermediate values.

of the same blood were kept on ice and aerated at various times after drawing, as indicated in Table II.

An inspection of Table II will show that there is a slight increase in the ammonia content after standing 30 minutes, but that further standing up to 3 or 4 hours causes no increase in the ammonia. Our results here are in striking disagreement with the conclusions of Folin and Denis (1), Barnett (35), and others, concerning the presence of "labile ammonia-yielding bodies" in blood.

It will be noted however, from Table II, that the first period of standing always yields a small increment of ammonia over that obtained when the blood is freshly aerated. Investigating this

TABLE I.  
*Recovery of Known Amounts of Ammonia Added to Water.*

NH <sub>3</sub> -N per 100 cc.		NH <sub>3</sub> -N per 100 cc.	
Taken.	Found.	Taken.	Found.
mg.	mg.	mg.	mg.
0.02	0.03	0.06	0.06
0.02	0.03	0.07	0.08
0.03	0.04	0.10	0.10
0.03	0.04	0.20	0.20
0.04	0.035	0.40	0.40
0.04	0.07	0.40	0.40
0.05	0.05		

question further, we carried out simultaneous analyses of dog blood and plasma under identical conditions.

From the results given in Table III it will be seen that with plasma there is no appreciable yield of ammonia after the first aeration, while just the opposite result is obtained with corpuscles.

Henriques and Christiansen (31) found that, on a volume basis, blood corpuscles contain from two to four times as much ammonia as the plasma. We believe from our results, however, that the concentration of ammonia is essentially the same in corpuscles and plasma; and we attribute the small increase which we find in blood on standing to hemolysis and perhaps to diffusion of ammonium salts from the corpuscles into the plasma.

To test whether ammonia would be split off from a typical protein under the conditions of our aeration we have carried out

the following experiments. Fresh hen's egg albumin was diluted with ammonia-free water, and 5 cc. portions were aerated immediately after addition of 1 cc. of carbonate-oxalate solution,

TABLE II  
*The Effect of Standing on the Blood Ammonia.\**

Subject.	Source of blood.	Repeated aeration of a single portion of blood after addition of carbonate-oxalate, and standing at room temperature.				Blood after standing on ice previous to addition of carbonate-oxalate.	
		Interval between drawing blood and first aeration.	NH <sub>3</sub> -N			Time of standing.	NH <sub>3</sub> -N
			First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.		
		min.	mg.	mg.	mg.	min.	mg.
Cat.	Renal vein.	15	0.18	0.05	0.04		
"	Vena cava.	5	0.12	0.06	0.07		
Dog.	Carotid.†	60	0.14	0.02	0.02	150	0.16
"	"	1	0.06			120	0.08
"	Renal vein.	15	0.09	0.02	0.00	120	0.14
"	Vena cava.	1	0.03	0.01	0.00		
"	" "	1	0.16	0.04	0.00	120	0.18
"	Carotid.	1	0.10			150	0.11
"	"	150	0.11	0.03	0.01		
"	Renal vein.	30	0.19			180	0.18
"	" "	10	0.18	0.04	0.01	240	0.28
"	Vena cava.	2	0.07	0.01	0.00	240	0.10
"	Renal vein.	180	0.18	0.03	0.00		
"	Femoral artery.	15	0.04	0.01	0.00	210	0.07
"	Carotid.	1	0.07			120	0.07
"	Renal vein.	15	0.21	0.03	0.01		
"	Vena cava.	5	0.08	0.01	0.00		
"	Carotid.	1	0.08			120	0.10
"	"	120	0.10	0.02	0.01		
"	Renal vein.	10	0.28	0.03	0.01		
"	Vena cava.	5	0.06	0.03	0.01	90	0.08

\* Results are given for 100 cc. of blood.

† This blood stood at room-temperature throughout.

again after 30 minutes, and again after a further 30 minute interval. The results are given in Table IV.

The figures reported in Table IV show that egg albumin does not decompose to yield ammonia under the conditions which



we used for blood. These results lend some support to the view that the quantities of ammonia found in blood are preformed, and are not decomposition products formed during the analysis. This latter view remains, however, a possibility.

TABLE III.

*Ammonia in Whole Blood and Plasma: Effect of Standing.*

Dog.	Source of blood.	NH <sub>3</sub> -N in 100 cc.					
		Blood.			Plasma.		
		First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.	First aeration.	Second aeration 30 min. after first.	Third aeration 30 min. after second.
		mg.	mg.	mg.	mg.	mg.	mg.
1	Vena cava.	0.18	0.05	0.00	0.20	0.00	0.00
2	Carotid.	0.11	0.03	0.01	0.13	0.00	0.00
3	Femoral artery.	0.07	0.03	0.00	0.08	0.01	0.00
4	Mixed systemic.	0.12	0.03	0.01	0.10	0.02	0.00
5	Carotid.	0.10	0.02	0.01	0.12	0.00	0.00

TABLE IV.

*Experiment 2. Ammonia in Egg Albumin Mixtures.*

No.	NH <sub>3</sub> -N in 100 cc.			Remarks.
	First aeration.	Second aeration 30 min. after first	Third aeration 30 min. after second.	
	mg.	mg.	mg.	
1	0.05	0.00		26 cc. of fresh egg albumin diluted to 44 cc.
2	0.00		0.01	Fresh egg albumin diluted four times.
3	0.07	0.00	0.00	" " " " to double volume.
4	0.03	0.00	0.00	" " "

The next point studied was concerned with the question as to whether minute amounts of ammonia added to shed blood can be recovered quantitatively. This question is directly related to the broader question as to whether ammonia may be transported in the blood in a complex combination from which the ammonia cannot be liberated by simple treatment with carbonate-

oxalate mixture. While this latter question is obviously difficult to study directly, it seems probable that if the blood possesses the power of combining ammonia, then minute amounts of ammonia added to blood should not be completely recovered.

In connection with any theory of a complex ammonia combination in blood it should be borne in mind that any combination of ammonia from which the base could not be liberated by treatment with sodium carbonate would presumably defeat the object of ammonia formation in the organism, if we assume that the ammonia is produced for the purpose of acid neutralization.

In testing the recovery of added ammonia we made use of ox blood. Simultaneous analyses were carried out with and without the addition of small amounts of ammonia nitrogen.

The figures given in Table V show that ammonia added to blood can be completely recovered within the limits of accuracy of the method.

The next experiment was planned to find out whether the blood can yield ammonia to neutralize added acid. Ammonia in such a combination would be readily available for the needs of the organism. The following typical experiment shows that blood does not yield ammonia to neutralize added acid.

Fresh ox blood gave on analysis 0.08 mg. of ammonia nitrogen per 100 cc. To 10 cc. of this blood was added 1 cc. of ammonia-free isotonic saline solution, and to a second 10 cc. portion of blood was added 1 cc. of a 10 per cent lactic acid solution. The two mixtures were then incubated in a water bath at 38–40°C. At the end of 30 minutes the blood-saline mixture gave 0.08 mg. of  $\text{NH}_3\text{-N}$  per 100 cc., and the blood-acid mixture gave 0.09 mg. of  $\text{NH}_3\text{-N}$  per 100 cc. 30 minutes later the values were 0.09 and 0.08, respectively.

A study of the blood ammonia concentration in animals where the urinary ammonia output is higher than normal was made upon phlorhizinized dogs which were available from other experiments. These dogs had received daily injections of 1 gm. of phlorhizin in oil over long periods, and blood was taken for analysis when the animals were in the late stages of the poisoning.

The results given in Table VI show that even where the ammonia content of the urine is markedly increased, there is no increase in the ammonia content of the blood. (Compare figures on normal dogs, Table II.)

TABLE V.

*Recovery of Ammonia Added to Blood. Results Given in Terms of  $\text{NH}_3\text{-N}$  per 100 cc. of Blood.*

No.	Original.*	Added.	Found.	Difference.	Remarks.
	mg.	mg.	mg.	mg.	
1	0.08	0.15	0.23	0.00	Added $\text{NH}_4\text{OH}$ . Aerated immediately.
1	0.075	0.075	0.18	0.03	“ “ “
1	0.075	0.15	0.23	0.005	Added $\text{NH}_4\text{Cl}$ . Aerated immediately.
2	0.20	0.20	0.37	0.03	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Aerated at once.
2	0.20	0.20	0.39	0.01	Added $\text{NH}_4\text{Cl}$ in isotonic saline solution. Aerated at once.
2	0.20	0.20	0.33	0.07	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
2	0.20	0.20	0.37	0.03	Added $\text{NH}_4\text{Cl}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
2	0.26	0.20	0.37	0.09	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 2 hours at room temperature before aeration.
2	0.33	0.20	0.45	0.08	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 24 hours in ice box before aeration.
3	0.20	0.20	0.38	0.02	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Aerated at once.
3	0.20	0.20	0.42	0.02	Added $\text{NH}_4\text{OH}$ in isotonic saline solution. Stood 1 hour at room temperature before aeration.
4	0.36	0.25	0.66	0.05	Added $\text{NH}_4\text{OH}$ . Stood 30 minutes at room temperature before aeration.
4	0.36	1.00	1.41	0.05	Added $\text{NH}_4\text{Cl}$ . Stood 30 minutes at room temperature before aeration.
5	0.09	0.094	0.20	0.016	Added $\text{NH}_4\text{-lactate}$ in isotonic saline solution. Aerated at once.
5	0.09	0.188	0.26	0.018	“ “ “

TABLE V--*Continued.*

No.	Original.*	Added.	Found.	Difference.	Remarks.
	mg.	mg.	mg.	mg.	
5	0.10	0.094	0.22	0.026	Added NH <sub>4</sub> -lactate in isotonic saline solution. Stood 30 minutes at 38-40° C. before aeration.
5	0.11	0.094	0.22	0.016	Added NH <sub>4</sub> -lactate in isotonic saline solution. Stood 1 hour at 38-40° C. before aeration.

\* The values in this column do not represent the average preformed ammonia nitrogen in fresh ox blood. The blood used was brought to the laboratory from a slaughter house, and the higher values found undoubtedly are to be attributed to bacterial action or to the presence of foreign material. We have found as the average value for 15 slaughter house bloods, when the analyses were made as soon as received, 0.13 mg. of ammonia nitrogen per 100 cc., but the conditions of handling and drawing this blood were not rigidly controlled.

TABLE VI.

*Experiment 6. Ammonia in the Blood of Phlorhizinized Dogs.*

Dog.	Source of Blood.	NH <sub>3</sub> -N in 100 cc. of blood.	NH <sub>3</sub> -N of total N in previous 24 hour urine.	Plasma CO <sub>2</sub> c. p.
		mg.	per cent	vol. per cent
6	Femoral artery.	0.075	11.0	
9	Carotid.	0.07	6.3	38.6
23	Femoral artery.	0.07	6.5	47.6
26	Jugular.	0.09	4.0	38.1
27	"	0.05	4.6	54.1

It seemed desirable next to test the question as to whether there is accumulation of ammonia in the blood following double nephrectomy, or after ligation of both ureters. Similar experiments have been reported for dogs by Winterberg (41) and for goats by Henriques and Christiansen (31). These investigators failed to find increased ammonia in the blood under the conditions cited. Since with the kidneys extirpated (or after ligation of both ureters) acid formation must be going forward, while ammonia cannot leave the organism, we deemed this question of sufficient import-

ance to warrant full repetition of the experiments on dogs. Our experiments in this connection were carried out as follows.

The dogs of this series were operated under ether anesthesia. Through a midline incision in the abdominal wall the renal vessels were ligated and the kidneys removed; in other cases only the ureters were tied off. We observed that those dogs, in which only the ureters were ligated and the kidneys left, survived the operation somewhat longer than those dogs whose kidneys were extirpated.<sup>6</sup> No convulsions or other typical "uremic" symptoms were observed in the operated animals other than slight tremors in the extremities, rapid and shallow breathing, and rapid and labored heart action. Blood was taken for analysis in most animals when it appeared that the animal was near death. In the oxalated blood the corpuscles settled with extraordinary rapidity, leaving a plasma of light yellow color.

An inspection of the results given in Table VII shows (in agreement with the previous investigators above cited) that in spite of total absence of kidney function in dogs there is no accumulation of ammonia in the blood. In fact some of these experimental animals gave us the lowest figures for ammonia in the blood which we have found. This failure to find any accumulation of ammonia in the blood after extirpation of the kidneys is, we believe, a unique finding for this substance as compared with any other constituent of both blood and urine, and we believe that this finding necessitates the conclusion drawn later in this paper concerning the origin of ammonia in the organism.

In view of the facts shown above, especially the findings that there is no accumulation of ammonia in the blood in phlorhizinized dogs, or in dogs without functioning kidneys, we were led to the conclusion that the kidneys themselves must produce the urinary ammonia. That the kidneys may perform an active synthetic function is not a new idea (Bunge and Schmiedeberg, 43), but so far as we know the production of ammonia has not hitherto been ascribed to the kidney.

It seemed probable that if ammonia production takes place in the kidney, this organ would not excrete every trace of the ammonia formed, and we might then expect to find the blood of

<sup>6</sup> In this we do not agree with Jackson (42).

the renal vein richer in ammonia than the systemic blood. We have therefore carried out experiments in which we compared the ammonia content of the carotid blood with that of the renal vein. In most cases we also included determinations of the ammonia content of blood from the vena cava taken posterior to where the renal veins enter this vessel.

In collecting the several bloods for analysis in this experiment we have proceeded as follows: The animal was anesthetized with ether, and a cannula placed in the carotid artery. In some cases the arterial blood was drawn first, in other cases last; we have not

TABLE VII.

*Experiment 7. The Blood Ammonia in Nephrectomized Dogs, and in Dogs with Ligated Ureters.*

Dog.	Source of blood.	Time after operation.	Per 100 cc. of blood.		Plasma CO <sub>2</sub> C. P.	Remarks.
			NH <sub>3</sub> -N	N-P-N		
		hrs.	mg.	mg.	vol. per cent	
7	Femoral artery.	44	0.06	200		Kidneys extirpated.
8	" "	46	0.08	182		Ureters tied.
8	" "	71	0.10	197		" "
14	" "	42	0.03	172	38.2	Kidneys extirpated.
18	Carotid.	47	0.03	162	57.1	Ureters tied. Autopsy disclosed severe hemorrhage due to bursting of capsule of one kidney.
21	"	47	0.12	186	36.2	Ureters tied.

found that the order of taking the blood is material. The abdomen was opened by a midline incision, and the vena cava and the renal vein on one side exposed. Blood was then taken from the renal vein (we found the most convenient method of taking this blood was by the use of a curved needle attached by a piece of rubber tubing to a 25 cc. pipette containing potassium oxalate, the blood being drawn into the pipette by carefully regulated suction). The point of the needle was introduced into the vein toward the kidney. The renal vein was tied off (to prevent hemorrhage), and a ligature placed around the vena cava just behind the renal veins. Blood was now quickly drawn from the vena cava, caudad

to the ligature, using a similar technique to that described for the renal vein.

It will be noted from the experiments reported in Table VIII that the blood of the renal vein is invariably much higher in ammonia content than the systemic arterial or venous blood.

TABLE VIII.

*Experiment 8. Comparison of Ammonia Content of Systemic and Renal Venous Blood.*

Subject.	Sex.	NH <sub>3</sub> -N in 100 cc. of blood.			Remarks.
		Carotid.	Vena Cava.	Renal vein.	
		mg.	mg.	mg.	
Cat 1	Male.	0.08		0.20	Ether anesthesia.
" 2	Female.	0.08		0.26	Chloretone anesthesia. Cat pregnant.
" 3	Male.	0.12	0.12	0.22	Ether anesthesia.
" 4	Female.	0.11	0.10	0.27	" " Cat pregnant.
" 5	Male.	0.12	0.12	0.18	Ether anesthesia.
Average.....		0.102	0.113	0.226	
Dog 10	Female.	0.08		0.22 (L) 0.18 (R)	Ether anesthesia.
" 11	Male.	0.06	0.03	0.09	" "
" 12	"	0.075	0.16	0.12	" "
" 13	"	0.10	0.07	0.19 (R) 0.18 (L)	" "
" 15	"	0.07	0.08	0.21	" "
" 24	Female.	0.14		0.25	" " Dog in last stages of phlorhizin poisoning.
" 25	"	0.13		0.18	" " "
" 27	"	0.05*		0.14	" " "
Average.....		0.088	0.085	0.176	

\* Blood of jugular vein.

The blood from the renal vein averages twice as much ammonia as does the blood from other sources.

These differences are so marked as to admit of only one interpretation; *viz.*, that the kidney, instead of excreting ammonia from the blood, forms the ammonia which it excretes, while at

the same time it contributes a small amount of ammonia to the blood. No theory of concentration (by abstracting water) can possibly explain the differences in ammonia content which we have found, since to explain the ammonia increase in the renal vein on such a basis we should have to assume a 50 per cent concentration of the blood in a single passage through the kidney.

As a corollary of the view that the kidney is the center of ammonia production in the body we should expect that acid or alkali introduction into the organism should have no detectable effect on the ammonia content of the systemic blood, while the ammonia content of the blood of the renal vein might be expected to show a slight increase in ammonia after acid introduction and a slight decrease after alkali ingestion. These results would be expected because as the kidney makes more or less ammonia we might expect some corresponding change in the ammonia escaping into the renal blood. There is no reason to believe that the slight changes which might be produced in the ammonia concentration of the blood of the renal vein should be reflected in the blood of the general circulation, for we must assume that the ammonia of the systemic blood represents an equilibrium state between the ammonia which comes into the circulation by way of the renal veins (and possibly traces of ammonia from the intestinal circulation which pass the liver) and the transformation of this ammonia into urea. If ammonia were formed in the organism in appreciable amounts elsewhere than in the kidney, we should expect injection of acid into the circulation to be followed by a definite increase in the ammonia of the general systemic blood. The opposite change might be expected as a result of alkali treatment. The experiments conducted in this connection were as follows.

#### *Effect of Acid Injection.*

The procedure was as follows: The animal was etherized and cannulae were placed in the carotid artery and jugular vein. A sample of carotid blood was taken and the ammonia determined. 1.0 N hydrochloric acid was then run from a burette into the jugular vein at the rate of about 1 cc. per minute, until the animal showed severe symptoms of dyspnea. As rapidly as possible then blood was again taken from the carotid artery, and from



the renal vein and vena cava. The ammonia in each of these bloods was determined, and in the carotid blood, the plasma  $\text{CO}_2$ -combining power was also determined.

*Effect of Alkali Injection.*

In one series of dogs we attempted to eliminate ammonia from the urine by means of sodium bicarbonate in olive oil injected subcutaneously. The urinary ammonia was determined in daily catheter specimens, and while it did not completely disappear it fell on several days to very low values. The ammonia and  $\text{CO}_2$ -combining power of the jugular blood was followed during the course of the injections, the specimens of blood being taken through a needle inserted through the skin into the vein.

TABLE IX.

*Experiment 9. Effect of Acid Injection on Blood Ammonia.*

Dog.	NH <sub>3</sub> -N in 100 cc. of blood.					Remarks.
	Before.	After.				
	Carotid.	Carotid.	Vena cava.	Renal vein.	Carotid plasma CO <sub>2</sub> C.P.	
	mg.	mg.	mg.	mg.	vol. per cent	
16	0.16	0.075	0.06	0.28	13.4	30 cc. of 1.0 N HCl injected.
17	0.075	0.085	0.10	0.25	18.5	45 cc. of 1.0 N HCl injected.

In one dog, blood taken from the jugular vein gave 0.07 mg. of ammonia nitrogen per 100 cc. The dog was then etherized and 2 per cent  $\text{NaHCO}_3$  solution run into the vein through a cannula. After 2 hours, when 196 cc. of the bicarbonate solution had been injected, the urine (by catheter) was ammonia-free. At this time, femoral arterial blood gave 0.07 mg., and renal venous blood 0.18 mg. of ammonia nitrogen per 100 cc.

From Tables IX and X it is plain that acid or alkali injection have no influence on the ammonia content of the systemic blood. There is some evidence of an effect on the ammonia content of the blood of the renal vein, but further experiments would be required to warrant any definite conclusion here.

TABLE X.

*Experiment 9. Effect of Alkali Injection on Blood Ammonia. NaHCO<sub>3</sub> in Olive Oil.*

Date.	Urine.		Blood.				NaHCO <sub>3</sub> injected.
	Reaction to litmus.	NH <sub>3</sub> -N	Jugular.		NH <sub>3</sub> -N in 100 cc. of renal venous blood.	NH <sub>3</sub> -N in 100 cc. of vena cava blood.	
			CO <sub>2</sub> c. p.	NH <sub>3</sub> -N in 100 cc.			

## Dog 19.

		per cent	vol. per cent	mg.	mg.	mg.	gm.
May 5	Basic.	0.038	47.7	0.10			2
" 6	"	0.010	60.0	0.05			3
" 11	"	0.115	52.9	0.07			2
" 12	"	0.030	*	0.06	0.13	0.08	

## Dog 22.

May 20	Acid.	0.152					2
" 23	Basic.	0.068	66.8	0.07			2.5
" 24	Acid.	0.050					
" 25	Basic.	0.040					2.5
" 26	"	0.036					4
" 27	"	0.004					4
" 28	"	0.014	80.2	0.07	0.16	0.06	

\* CO<sub>2</sub>-combining power of carotid blood plasma, under ether anesthesia, was 39.3 volumes per cent.

## DISCUSSION.

If we accept the view that ammonia production takes place in the kidney as part of its excretory function, it would seem that certain facts in regard to acidosis may be more readily understood. Under the commonly accepted view that neutralization of acids by ammonia is a function of the organism in general, or of the liver, it would seem very difficult to understand such acidosis (depletion of the alkali reserve) as frequently occurs in nephritis, where there is no marked increase in acid production. Even the increase in acid phosphate in the blood reported in some of these cases affords no explanation of depletion of the alkali reserve, since ammonia should be available for neutralization of any circulating acid.

If, however, we look upon the kidney as the seat of ammonia production, depletion of the alkali reserve becomes readily understandable under certain definite conditions. If ammonia is not available within the organism the acids must be transported wholly in combination with the fixed bases, or with protein. A depletion of the alkali reserve of the blood could therefore arise under any one of three definite conditions.

1. Introduction of acid radicles into the blood stream more rapidly than the normal kidney can eliminate them, or can make ammonia to combine with them while eliminating them.

2. If the kidney becomes defective in its power to eliminate acid radicles, and thus to maintain them at a minimal level in the blood, a depletion of the alkali reserve would result, since the acid radicles would remain in the circulation in abnormal amounts, and would have to be neutralized by the fixed bases or protein. This condition might well result with a kidney still normal in its power of ammonia production. Such ammonia is available for the needs of the organism only as acid radicles are excreted.

3. A depletion of the alkali reserve of the blood would result should the kidney become defective in its power of ammonia formation. Even should such a kidney remain normal in its power of excreting acid radicles, the organism would lose base excessively during the excretion of the acid.

It would appear that the first of these three forms of acidosis occurs, if at all, in diabetes. Very probably either or both of the two latter forms occur in nephritis. It seems from our results that acidosis in the sense of depletion of the alkali reserve is primarily a kidney disease.

Although Wakeman and Dakin (44) came to the conclusion that the formation of urea in the animal body is an irreversible process, we believe that urea is the most probable precursor of ammonia in the kidney. It has been frequently demonstrated that the urinary ammonia is increased at the expense of urea, and unless we assume a conversion of urea into ammonia by the kidney we should have to assume the transportation in the blood of some intermediate product between urea and ammonia, or of some "complex ammonia compound." Our work has rendered either of these views very improbable. It is, of course, also possible that the kidney is active in deamination of amino-acids, and that excreted ammonia is supplied from this source.

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# THE MECHANISM OF REDUCTION OF NITRATES AND NITRITES IN PROCESSES OF ASSIMILATION.

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The mechanism of reduction of nitrates of the alkali metals to ammonia and the formation of amino nitrogen in biochemical syntheses from inorganic nitrogen compounds have not been explained satisfactorily. Some investigators explain the transformation by assuming a direct reduction of the nitrates to ammonia by action of nascent hydrogen, while others assume an intermediate reduction of the nitrate to nitrite, from which, as the reduction proceeds, ammonia is produced. The exact chemical procedure by which bacteria or molds are able to produce ammonia or nitrites from alkali nitrates also has not been satisfactorily explained.

Schimper,<sup>1</sup> the botanist, has been able to demonstrate experimentally that the reduction of nitrates in green leaves is connected intimately in some manner, not only with the influence of light, but also with the action of iron compounds in the leaves. This observation was so interesting to the writer that it led him to an investigation of the question whether iron actually takes part in the reduction of nitrates by means of bacteria. This work has proved very productive and, as a matter of fact, experiments have shown conclusively that the *cholera bacillus*, which possesses extraordinary reducing power for nitrates, has the ability to accumulate iron<sup>2</sup> in its organism, and its reducing power may possibly be a function, not only of its ability to absorb oxygen by respiration, but also of its iron content.

The data revealed through this biological research, which was interrupted by the War and political disturbances in Europe,

<sup>1</sup> Schimper, A. F. W., *Bot. Z.*, 1890, xlv, 73.

<sup>2</sup> Unpublished data.

warranted a purely chemical investigation of the reduction of inorganic nitrates by means of iron salts. The opportunity to continue this work has now been offered to me, and, as a result, the study of this interesting problem has been taken up.<sup>3</sup>

An interesting paper by Menaul which recently appeared in this Journal,<sup>4</sup> brings up for discussion a very important biochemical change. This investigator describes the action of formaldehyde on saltpeter in aqueous solution when exposed to sunlight, and the observation is made by him that in such solutions small quantities of hydrocyanic acid can be detected easily. This quite remarkable action of sunlight on nitrates was observed by the writer several years ago<sup>5</sup> and this preliminary paper is now contributed to bring the results of this work before the American reader, and at the same time to present a summary of the principal results of his earlier investigations on the photochemical reduction of nitrates and the reduction of nitrates and nitrites with iron salts, which have appeared in various scientific publications during the last 10 years.<sup>6</sup> This summary will be presented in three parts as follows: (1) Reduction of nitrates of alkali metals by means of light and also iron salts; (2) Reduction of nitrites of alkali metals by means of light and also iron salts; and (3) Synthesis of organic compounds containing nitrogen from inorganic compounds of nitrogen.

### *The Reduction of Nitrates.*

In the course of researches on the photochemical decomposition and synthesis of nitrates and nitrites, the observation was made, for the first time, that one oxygen atom in nitrates must be bound in the molecule in a manner quite different from that of the oxygen atoms in nitrites. The writer has used the specific term "nitrate oxygen atom"<sup>7</sup> to designate that oxygen atom which

<sup>3</sup> Through cooperation with Prof. Treat B. Johnson, who has supplied rare research material, it has been possible to extend the field of investigation into the pyrimidine and purine series. The results of these researches, which are of immediate biochemical interest, will be published at a later date.

<sup>4</sup> Menaul, P., *J. Biol. Chem.*, 1921, xlv, 297.

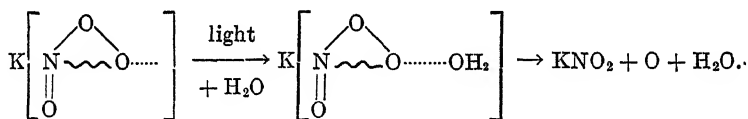
<sup>5</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1151.

<sup>6</sup> Baudisch, O., *Ber. chem. Ges.*, 1911, xlv, 1009.

<sup>7</sup> Baudisch, O., *Ber. chem. Ges.*, 1912, xlv, 2879; 1916, xlix, 1176.

is easily split off from such salts either under the influence of light or of metallic iron. That we are dealing here with such a labile oxygen linking in nitrates is demonstrated to us by nature, in that the various nitrifying and denitrifying bacteria have the power to differentiate between nitrate and nitrite oxygen. The question naturally arises, under what influence or by means of what power an atom of oxygen can be split out of potassium nitrate, for example, with the formation of a nitrite. This problem was first attacked from a purely chemical standpoint, but it is now proposed to continue the study also from a biological point of view.

In explanation of the photochemical reduction of nitrates in aqueous solution, we may assume, according to Werner's theory<sup>8</sup> of reaction, an activation of the residual valence of an oxygen atom of the nitrate and of the oxygen atom of the water, resulting in the attraction of molecules of water into the inner sphere of the nitrate molecule. There follows a dissociation of the nitrate molecule with the formation of oxygen as is expressed by the following equation:



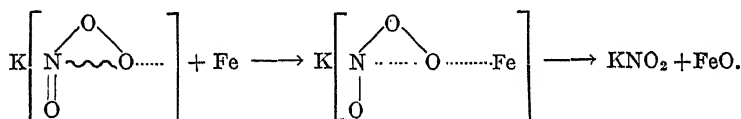
In other words, this reduction process takes place under the influence of light even in an atmosphere of oxygen, and stops at the nitrite stage. The reverse process can also take place and nitrite is readily formed from ammonia by photochemical oxidation without, however, any production of nitrate.

It is apparent from these facts that light can readily split off an atom of oxygen from nitrates of the alkali metals, without the presence or influence of either nascent or molecular hydrogen. This dissociation of nitrate into oxygen and nitrite can also be brought about by means of metallic iron as well as under the influence of the energy of light. If a neutral oxygen-free solution of potassium nitrate be shaken in a vacuum with active iron prepared by reduction with hydrogen, the supernatant liquor obtained

<sup>8</sup> Werner, A., *Neuere Anschauungen auf dem Gebiete der anorganische Chemie*, Brunswick, 4th edition, 1920.



after the iron powder has been allowed to settle will give every reaction applicable for the detection of nitrous acid.<sup>9</sup> In other words, metallic iron will easily reduce potassium nitrate to potassium nitrite in the cold in the absence of every trace of oxygen, and under conditions such that neither the action on the iron by water nor the effect of nascent hydrogen can possibly come into consideration. These results lead to the assumption, therefore, that iron readily splits off an oxygen atom of the nitrate after having first entered into a loose combination with it. This change may be expressed by the following equation:



From these examples it is seen that the photochemical reduction of nitrates to nitrites and their reduction by means of metallic iron are similar in nature, and in both cases there occurs either an activation or mobilization of the valence energy leading to the formation of an unstable addition product, which finally breaks down into the final products of reaction. Although it has been possible to show a relation between the photochemical reduction of nitrates to nitrites and the corresponding biological reduction in green leaves, it has not yet been possible to connect known chemical reduction processes with the biological reductions occurring naturally in bacteria or in molds.

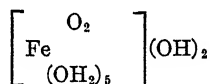
It formerly appeared scarcely possible to attach any biological importance to ferrous hydroxide in these reduction processes because, as was commonly believed, the reaction proceeded stoichiometrically and ferric hydroxide was formed at the expense of the oxygen split off from the nitrate. In biological processes, however, one cannot speak of stoichiometrical reactions in connection with metals, because, as is well known, they are present only in traces. Their action must be explained, on the contrary, by a particular energy inherent in their molecules, and consequently the writer has been accustomed to consider the metals as functioning in biological processes either as "mobilizers" or "catalysts,"

<sup>9</sup> Baudisch, O., *Ber. chem. Ges.*, 1921, liv, 406.

having the power to bring into play the latent energy of certain organic molecules.

From the point of view of Werner's coordination theory, it is possible to conceive of a relation between the mobilizing power of such a catalytic agent, and the peculiar power expressed by residual valency, which, as is well known, has the ability to draw new atoms, molecules, and radicals into the sphere of action of the internal nucleus of the metal, resulting in the most varied types of reactions. In fact, it is well known that extremely finely divided metals, such as platinum, palladium, or iron, possess these valence powers to a large degree, and their specific action has often been placed in parallel with purely biological processes.

The reduction of nitrates with ferrous hydroxide assumed a new interest when it was discovered that this reagent alone does not split off nitrate oxygen as had been assumed, but reduces nitrates only under the influence of oxygen.<sup>10</sup> As it is well known that white ferrous hydroxide is converted instantaneously into green ferrous hydroxide peroxide by the oxygen of the air, it is reasonable to say, therefore, that this polymolecular combination or peroxide is the active reagent which brings about this transformation of nitrates into nitrites.



(Coordination formula for ferrous hydroxide peroxide.)

The mechanism of this reduction has not yet been explained, but the attempt will be made here to show that the free energy of ferrous hydroxide is increased enormously by a loose combination with an oxygen molecule, and that this increase in energy makes itself apparent both physically and chemically. The striking effect on the color of white ferrous hydroxide, which is caused by the smallest trace of oxygen, shows that the oxygen enters into the inner sphere of the iron nucleus. Schäfer<sup>11</sup> has proved spectroscopically that such extraordinary alterations in color, either in the visible or in the invisible part of the spectrum, can only take place simultaneously with changes in the inner sphere

<sup>10</sup> Baudisch, O., *Ber. chem. Ges.*, 1921, liv, 410.

<sup>11</sup> Schäfer, K., *Z. anorg. Chem.*, 1918, lxxxvi, 221.

of the molecule. It has, however, not yet been shown how many oxygen molecules are present in such a molecular combination. It may be possible that the observation of Meyer,<sup>12</sup> who discovered that strongly magnetic substances were rich in absorption bands, whereas diamagnetic substances were poor in absorption bands, has some connection with these facts. This coincides completely with the action of the above mentioned iron compound, because, while the white ferrous hydroxide has practically no magnetic properties, the green to black ferrous hydroxide peroxide possesses magnetic properties which are almost equal to that of metallic iron. According to Hilpert,<sup>13</sup> when a stream of air or oxygen is led through a precipitate of ferrous hydroxide, the magnetic properties of the precipitate increase rapidly. Quartaroli<sup>14</sup> has also shown that oxidation with air converts the ordinary non-magnetic ferrous hydroxide into mixed ferro-ferri oxides, which possess a susceptibility almost a hundred times greater than ferric salts. In fact the magnetic susceptibility of  $\text{Fe}_3\text{O}_4$  approaches that of the metal iron itself. It may be concluded, therefore, from our present knowledge, that there is a very close relationship between the physical properties of metallic iron and those of ferrous hydroxide peroxide, and it will be of the greatest interest and importance to determine whether there is also any direct relationship between the peculiar chemical properties of this peroxide and those of finely divided metals. The simplest explanation, therefore, for the reduction of nitrates is the assumption that freshly precipitated, colloidal ferrous hydroxide peroxide acts catalytically as a finely divided metal.

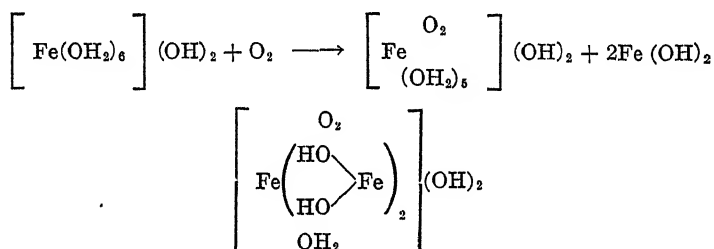
It seems very probable that the peculiar properties resulting from the colloidal nature of ferrous hydroxide peroxide and the properties of the metal resulting from its position as the central atom of a complex system actually coalesce. Furthermore, these characteristic properties apply only to the peroxide and not to ferrous hydroxide, because the latter compound is not only unable to bring about a reduction of nitrates, but also will not react to form polynuclear compounds. Not until brought under the influence of oxygen does the central iron nucleus of ferrous hy-

<sup>12</sup> Meyer, S., *Wied. Ann.*, 1899, lxxviii, 325.

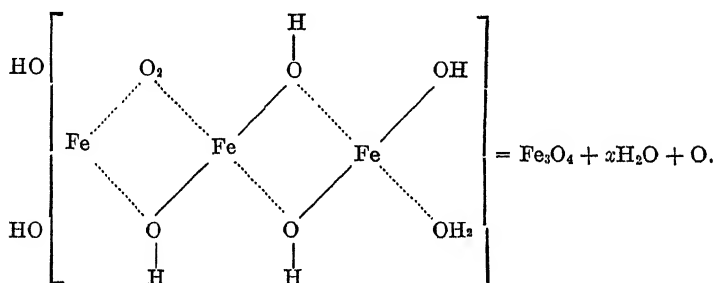
<sup>13</sup> Hilpert, S., *Ber. chem. Ges.*, 1909, xlii, 2248.

<sup>14</sup> Quartaroli, A., *Chem. Zentr.*, 1917, i, 729.

dioxide or other ferrous salts acquire the property of attracting new molecules of ferrous hydroxide into its inner sphere. This coordination combination exists, according to Werner, not between iron and iron, but between the active hydroxyl oxygen atoms of the ferrous hydroxide, which are attracted to the central iron nucleus of the peroxide by its residual valency. It is also possible for the iron atoms of ferrous hydroxide to be held in combination through the peroxide oxygen atoms, as has been demonstrated in the case of cobalt compounds by the classical researches of Werner. The mechanism of the autooxidation of ferrous hydroxide and the formation of strongly magnetic  $\text{Fe}_3\text{O}_4 \cdot x\text{H}_2\text{O}$  may be expressed by the following formulas:



The structure of this polynuclear combination may be expressed graphically as follows:



From these graphic representations it is seen that ferrous hydroxide is converted by the absorption of oxygen into a peroxide of greater potential energy, whose iron nucleus, as experience has shown, possesses the property of intensifying the activity of and of entering into loose combination with the residual valence of oxygen atoms in other molecules of ferrous hydroxide present.

With the coordination theory of Werner as a basis, it becomes apparent from the foregoing that ferrous hydroxide may be transformed by the absorption of oxygen into a complex salt whose iron nucleus, just as finely divided metals, may enter into a wide range of reactions. A nitrate oxygen atom may be split off as well by means of light as by means of metallic iron or by ferrous hydroxide peroxide. All three of these processes of reduction may be considered to depend upon the same principle; namely, the mobilization or activation of the energy in the residual valence of the reacting materials. Ferrous hydroxide peroxide reacts most probably in a very similar manner to finely divided iron or platinum.

#### *The Reduction of Nitrites.*

Aqueous solutions of potassium nitrite containing easily oxidizable substances, such as alcohols, aldehydes, sugars, starches, etc., suffer a comparatively rapid reduction and decomposition under the influence of diffused daylight, and the change may be expressed very simply as follows:



The presence of potassium nitrosyl in solution may be detected by means of its condensation reaction with aldehydes (Angeli's aldehyde reaction).<sup>15</sup> Hydroxamic acids are formed as products of reaction, and as is well known, these acids give characteristic complex salts with iron which are colored a deep reddish violet. This reduction of potassium nitrite to potassium nitrosyl can also be accomplished by means of complex iron salts. The smooth reduction of potassium nitrite *via* potassium nitrosyl to ammonia by means of glucose, in the presence of very small quantities of iron, possesses particular biological interest. The system, glucose + iron + alkali, which is a fundamentally new reducing combination, does not attack in the least the alkali salts of nitric acid.<sup>16</sup> It is therefore possible to make a quantitative separation

<sup>15</sup> Angeli, A., *Samml. Chem. u. chem. Techn. Vortr.*, 1908, xiii.

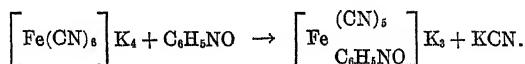
<sup>16</sup> A method, based on this observation, has been developed for the quantitative determination of nitrites and nitrates in the presence of other nitrogen compounds in soil extracts. Pfeiffer, T., and Simmerbacher, W., *Land. Versuchsstat.*, 1916, xciii, 65; *J. Soc. Chem. Ind.*, 1919, xxxviii, 507.

between nitrate and nitrite by means of a grape sugar-iron-alkali solution. Grape sugar, which is absolutely free from iron, does not cause the slightest reduction, even on heating, of nitrites; and also chrysarobin (1,8-dioxy-3-methyl-anthranol), which occurs quite widely in the vegetable kingdom, has not the ability of reducing the salts of nitrous acid. On the other hand, the addition of traces of any iron salt to an alkaline solution of either of these substances enables them to reduce immediately the nitrites by way of nitrosyl to free ammonia. The particular part that iron plays in these reactions remained a mystery for a long time, but seems now to have been explained quite satisfactorily.

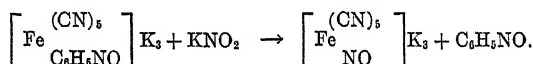
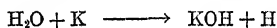
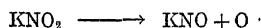
To explain this reduction process and its application for the quantitative separation of nitrates and nitrites, we may assume that the unsaturated, trivalent nitrogen atom of the nitrite molecule enters, through its activated residual valency, into a loose combination with the central iron nucleus of whatever complex salt is present, and then dissociates through the intermediate formation of nitrosyl into NO and K. The residual valency of the nitrate oxygen atom is not sufficiently active or powerful to displace the molecules or radicals already present in the inner sphere of the iron nucleus, and therefore these compounds are not attacked. That these reductions may all be considered as complex salt reactions, or in other words "nuclear exchange or displacement reactions" may be shown by the following example: A solution of 1 gm. of  $K_4Fe(CN)_6$ , 1 gm. of  $NaNO_2$ , and 5 gm. of sodium carbonate in 200 cc. of water, is distilled in a stream of oxygen. After interrupting the stream of oxygen, it is possible to detect nitrous acid in the distillate. The oxygen, under the influence of heat, has displaced the cyanogen group from the inner sphere, and in its place a molecule of nitrite has entered. The nitrite, however, decomposes and its scission product, NO, which at first takes its place in the inner sphere of the iron nucleus, is in turn displaced by the oxygen, and finally passes over into the distillate where it is easily detected as nitrous acid.

The fact that this remarkable reaction may not only be influenced by daylight, but in some cases will not take place except under the influence of daylight, is of particular chemical and biological interest. For example, if a freshly prepared solution of potassium ferrocyanide be treated with an aqueous alcohol

solution of nitrosobenzene and placed under the influence of diffused daylight, the solution which in the beginning possesses a weak, greenish yellow color, changes in a few minutes to a deep reddish violet. The mechanism of the reaction may be expressed as follows:



On treating the aqueous solution of this reddish violet compound with an excess of potassium nitrite and again placing the solution under the influence of diffused daylight the following decompositions take place:



The deep violet color of the solution disappears very rapidly and the reddish yellow or potassium nitroprusside takes its place. By means of this reaction, the reduction of sodium nitrite by means of a complex iron salt and light is demonstrated.

These processes of reduction of nitrites by way of nitrosyl to ammonia, may be drawn into intimate relation with biological reductions of nitrite, particularly as sugar, or its products of decomposition; and iron constantly accompany the nitrates in plants or in bacteria. Kostyschew and Tswekowa<sup>17</sup> state that the reduction of nitrate to nitrous acid takes place without the presence of any sugar, but that the further conversion of the nitrous acid, at least in the case of *Mucor racemosus*, is accomplished only in the presence of sugar. It seems likely, from investigations with cholera bacteria, that nitrates are reduced to nitrites by way of nitrosyl, because it was possible to detect the alkaline decomposition products of nitrosyl, for example, NO and NH<sub>3</sub> (the latter as a reduction product of NO), in the volatile portions of alkaline cholera peptone cultures.<sup>18</sup>

<sup>17</sup> Kostyschew, S., and Tswekowa, E., *Z. physiol. Chem.*, 1920, cxv, 171.

<sup>18</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1148.

The assumption that nitrite is converted into nitrosyl finds a further support in the qualitative and quantitative composition of the gases which are produced during the photochemical reduction of nitrites in the presence of formaldehyde and during the biological reduction. For example, those bacteria which have the property of decomposing nitrates, produce a fermentation gas which consists of about 65 to 72 per cent  $N_2O$ .<sup>5</sup> In addition to the nitrous oxide, there is also always formed a little nitrogen, traces of NO, and also of prussic acid. A formaldehyde-potassium nitrite solution produces a gas under the influence of diffused daylight, which contains 64 per cent  $N_2O$ , as well as very small quantities of NO and HCN. The latter two gases were detected qualitatively by sensitive reactions. Clawson and Young<sup>19</sup> have detected prussic acid in cultures of *Bacillus pyocyaneus* and of other bacteria.

To summarize, the photochemical reduction of the alkaline nitrites proceeds by way of the reactive intermediate product, potassium nitrosyl, which may be detected by means of aldehydes, as well in the case of the photochemical reduction of nitrites as in the case of a reduction with the system, grape sugar + iron + alkali. Certain complex iron salts possess the property of reducing nitrites, whereas under these conditions nitrates remain unchanged. The reduction of the alkali nitrites by means of complex iron salts depends most likely upon the residual valency of the central iron nucleus and in all these changes light as well as heat exercises a very fundamental influence.

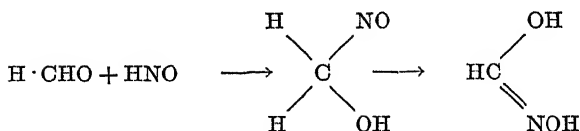
*Synthesis of Organic Compounds Containing Nitrogen from Inorganic Compounds of Nitrogen.*

Nitrosyl, which is formed in the reduction of alkali nitrates, interacts readily with formaldehyde with formation of formhydroxamic acid (Angeli's aldehyde reaction). This reaction proceeds, as was shown by the writer and Coert,<sup>20</sup> through the intermediate formation of nitroso methyl alcohol. Formhydroxamic acid is then formed from this by molecular rearrangement, and may be detected easily by means of its characteristic iron and copper salts, which are highly colored.

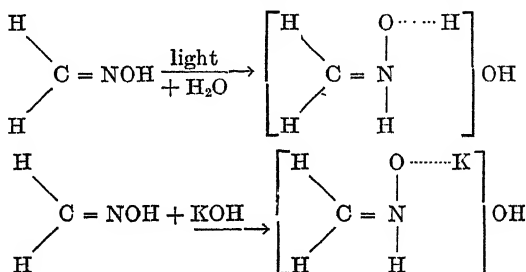
<sup>19</sup> Clawson, B. J., and Young, C. C., *J. Biol. Chem.*, 1913, xv, 419.

<sup>20</sup> Baudisch, O., and Coert, J. H., *Ber. chem. Ges.*, 1912, xlv, 1775.

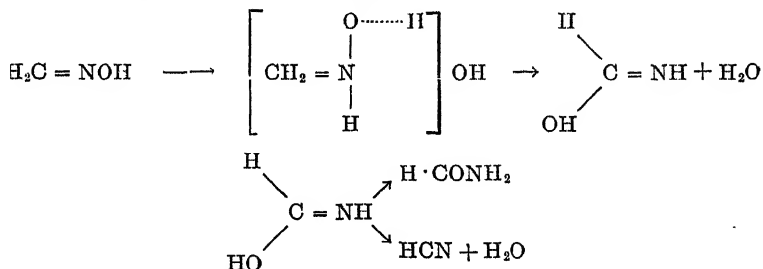




Formhydroxamic acid, under the influence of light, loses an atom of oxygen and is converted into formaldoxime, which is also characterized by its great reactivity. The stable form of this compound is altered under the influence of light and also alkali, and is transformed into an extremely labile modification. This transformation may be expressed as follows:<sup>21</sup>

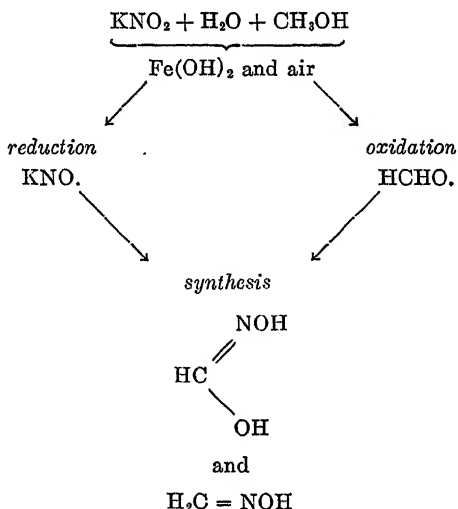


As is well known, formaldoxime exhibits a strong tendency to polymerize with the formation of three-carbon chain compounds. Its labile form is also capable of reacting further with aldehydes with the formation of three-carbon compounds, and furthermore under the influence of light combines with formaldehyde to form cyclic combinations containing both nitrogen and carbon. Under the influence of light formaldoxime undergoes, in part, a Beckmann rearrangement with formation of formamide, and, in part, a complete dissociation into prussic acid, water, and ammonia. These changes are expressed below:



<sup>21</sup> Baudisch, O., *Ber. chem. Ges.*, 1916, xlix, 1159.

The small quantities of prussic acid that are always found accompanying the treatment of formaldehyde in nitrate solutions with light, or by the reduction of nitrates with bacteria, may have been formed in accordance with the above reaction from the aldoximes. The photochemical formation of nitroso methyl alcohol (or hydroxamic acid) from formaldehyde, methyl alcohol, and nitrosyl may be the chemical counterpart of a possible biochemical formation of carbon-nitrogen-containing organic substances from inorganic nitrogen. The next step to amino nitrogen is simpler and may either take place by reduction or, as in the case of aldoxime, by simple rearrangement. It seems probable that the proof of the biological importance of nitroso methyl alcohol or formhydroxamic acid is found in its marked reactivity and in the pronounced tendency which it has to rearrangement, to polymerization, and to the formation of complex salts, particularly of iron. It seems extremely possible to introduce amino nitrogen into the higher alcohols, sugars, starches, etc., by means of nitrosyl which is formed photochemically from the nitrites and which is capable of entering into such widely different reactions.



Recent work<sup>10</sup> has revealed the fact that light may be replaced in certain cases by means of ferrous hydroxide and oxygen. Fer-

rous hydroxide peroxide oxidizes alcohols to aldehydes simultaneously with the reduction of nitrites to nitrosyl, and therefore synthesis of formhydroxamic acid or formaldoxime takes place. In fact, in these solutions of ferrous sulfate, containing bicarbonate and nitrite, three dissimilar reactions proceed simultancously—oxidation, reduction, and synthesis.

To summarize, nitrosyl is formed from alkali nitrites photochemically and by reduction with glucose in the presence of iron and by reduction with ferrous hydroxide in the presence of oxygen. The formation of carbon and nitrogen organic compounds in green plants and bacterial cultures from inorganic nitrogen, and the production of  $N_2O$ ,  $N_2$ ,  $NO$ , and  $HCN$  during fermentation and photochemical reduction may be explained by the intermediate formation of nitrosyl  $H\{NO^{22}$  and its subsequent reaction with aldehydic combinations.

<sup>22</sup> Bracket indicates labile character.

# STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

## VII. THE INFLUENCE OF VARIOUS PROTEIN SPLIT PRODUCTS ON THE METABOLISM OF FASTING DOGS.

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(Received for publication, August 1, 1921.)

It is the design of this study to extend certain recently developed aspects of the problem of proteose intoxication.

The toxic properties of protein split products, at the proteose stage of hydrolysis, have called forth a wide literature (1). This has been primarily concerned with the clinical effects of a rapid intravenous injection of a concentrated solution of proteose. The chief signs emphasized were, the depression of blood pressure and respiration, the delayed coagulability of the blood, the lymphagoguic effect, the constitutional depression or "peptone shock," the gastrointestinal irritation, the diminished response to subsequent injections or "immunity," the leucopenia, the marked concentration of the blood,<sup>1</sup> the severe acidosis,<sup>1</sup> and the rapidly fatal issue. These constitute a syndrome rivalling in violence any that we know. Naturally proteoses were assigned the toxic agency in diseases where injury of tissue was a factor. In the absence of a more tangible cause it was widely assumed that "the absorption of toxic proteoses" explained an acute intoxication. Recently, these assumptions have been given experimental ground.

Vaughan (2) and his coworkers have prepared toxic split products from a variety of proteins possessing many of the properties of proteoses. They concluded from their experiments that all

<sup>1</sup> Author's unpublished data.

proteins contain a toxic nucleus which when released gives rise to an intoxication identical with an infectious process. They have offered an attractive theory of infection and immunity on the basis of this work.

Biedl and Kraus (3) showed the physiological resemblance between peptone shock<sup>2</sup> and anaphylactic shock, and urged the view that the formation of digestion products identical with those present in the market peptone were responsible for anaphylactic shock. Indeed the mechanism of anaphylactic shock as presented in the work of Jobling, Peterson, and Eggstein (4) is essentially a proteose intoxication, by proteose derived from the host's serum as a result of the disturbance of the ferment antiferment balance. And more to our point Whipple and Cooke (5) and their coworkers in a series of extensive experiments have demonstrated that the intoxication of intestinal obstruction in dogs is due to the absorption of a toxic proteose produced in the obstructed gut. They have isolated and purified a proteose and clinically reproduced the disease by injection of the substance in normal dogs. Moreover they have shown that such injections cause a very large increase in the output of urinary nitrogen and a significant rise in non-protein nitrogen of the blood.

But they have gone further and it is with this phase that the present work is concerned. In a series of papers on proteose-intoxications and injury of body protein (6) these authors showed that inflammatory processes initiated either through bacterial agency or by aseptic means also call forth an increase in urinary nitrogen excretion and blood non-protein nitrogen. Hence they offer the suggestion that perhaps every inflammatory process is fundamentally a proteose intoxication. According to this view an inflammatory process may so injure body cells that toxic proteoses are formed which injure other cells, etc., so that a vicious circle is established. This hypothesis is certainly highly suggestive and if correct would render all inflammatory reactions simple of explanation and reduce the whole process to a single simple reaction.

We have been working for some time on proteose intoxication and have been particularly interested in these view-points. We have asked ourselves first of all whether the catabolic reactions called forth by Whipple's toxic proteose are specific or whether

<sup>2</sup> Referring to commercial "Witte pepton" composed of a mixture of proteoses and peptone.

such reactions are common to all proteoses. And, secondly, are there any other protein split products that are likely to occur in tissue injury capable of the same catabolic effect?

### *Technique.*

The plan of the experiments was to follow the urinary nitrogen, creatine, and phosphorus in fasting dogs until a nitrogen level had been reached, to inject intravenously a solution of the substance under consideration in normal saline solution, then to continue the experiment until these excretory products had returned to normal. Usually 4 days of fasting sufficed to bring the dogs to a basal nitrogen level. On the 4th day the injection was made into the jugular vein, under light ether anesthesia. The rate of injection was usually slow enough to avoid acute shock (in 5 to 15 minutes). The preparations will be described in their places.

The animals were kept in standard metabolism cages and the urine collected by catheterization every 24 hours. They were allowed to drink water according to their desire. The dogs were usually house-broken so that we had little trouble from contaminations of the urine with feces or vomitus. When such occurred, they were included in the 24 hour samples. Nitrogen was done by the Kjeldahl method, creatine and creatinine by Folin's methods, and total phosphorus by the uranium acetate method. We confined ourselves to these urinary constituents because we found early that they were the only ones to show any significant change. The clinical effects were typical and are not recorded because they have been sufficiently described elsewhere.

### *Control Experiments.*

In Table I and Chart 1 are given the results of several control experiments. Dog 1 was fasted for 7 days. It will be observed the constituents maintain a comparable level. Dog 2 received an intravenous injection of normal saline solution under identical conditions with the other experiments. The technique employed apparently has no influence on the course of metabolism. In Dogs 14, 15, and 16 we tried to show that the injection of a colloidal solution was in itself unable to affect

metabolism. This was of importance, because most of the substances subsequently injected were colloidal in character. The

TABLE I.

*Influence of Fasting, Injection of Normal Saline Solution, and Non-Protein Colloids.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
1	2		90	1.890	20	350	Fasting only.
	3		80	1.830	35	362	
	4		70	1.830	49	400	
	5		73	1.850	62	387	
	6		88	1.710	54	312	
	7		72	1.740	40	375	
2	5	8.5	66	3.020	29	340	Injected 50 cc. of normal saline solution.
	6		57	2.880	35	300	
	7		60	3.030	26	359	
	8		56	2.920	14	347	
	9		54	2.780	14	385	
	10		53	2.710	30	341	
14	3	15.0	74	3.380	45	490	Injected 50 cc. of solution of soluble starch.
	4		90	3.250	51	580	
	5		78	3.040	29	440	
	6		74	3.250	50	438	
	7		72	2.960	22	420	
15	4	10.4	110	3.620	102	663	Injected 30 cc. of strong solution of inulin.
	5		120	3.330	117	675	
	6		90	2.840	81	525	
	7		100	2.640	83	565	
16	4	7.8	90	2.950	20	338	Injected 50 cc. of 6 per cent gum acacia solution.
	5		92	2.205	4	337	
	6		96	1.890	11	312	
	7		92	1.890	18	262	
	8		72	1.830			

influence of the physical state of the solution injected is negative, from our view-point.

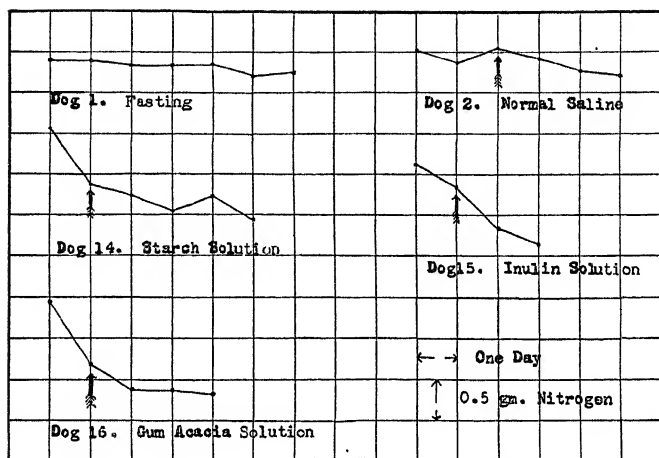


CHART 1. Control experiments. Influence of fasting, the injection of normal saline solution, and of non-protein colloids, on the daily excretion of nitrogen. Arrows show time of injection.

### *Effect of Amino-Acids, Amines, and in Vitro Autolysates.*

At various times it has been claimed that specific amino-acids are responsible for the toxic effects of protein split products (7). This contention has been disposed of as far as the clinical effects are concerned (1). What is the influence of free amino-acids on tissue catabolism?

In Table II and Chart 2 are given the results of the injection of various free amino-acids and mixtures. Dogs 17, 18, and 19 received pure amino-acids, leucine, glycocholl, and alanine. The results are entirely negative. The amount of nitrogen injected is excreted, but no more. In Dog 18 the creatine showed a marked rise, but this effect could not be repeated in Dog 19. We may therefore disregard it. Dogs 20 and 21 received a mixture of amino-acids prepared from the hydrolysis of casein until biuret-free. These preparations contained most of the important amino-acids yet were entirely innocuous. It might be argued that the responsible amino-acid was not present in sufficient concentration. But it will be seen that one-half the amount of nitrogen here injected, also derived from casein but given in the form of a pro-



TABLE II.

*Influence of Amino-Acids, Histamine, and in Vitro Autolysate.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		kg.	cc.	gm.	mg.	mg.	
17	2		72	2.330	72	400	
	3	9.6	44	2.150	26	345	
	4		70	2.240	24	430	Injected 50 cc. solution of glycocoll (0.288 gm. N).
	5		34	1.400	0	240	
	6		40	1.692	25	240	
	7	9.0	48	1.746	0	220	Injected 50 cc. solution of leucine (0.270 gm. N).
	8		41	1.960	25	220	
	9		50	1.600	7	230	
18	2		90	2.272	80	440	
	3	12.7	68	1.892	0	500	
	4		130	2.552	8	520	Injected 100 cc. solution of alanine (0.75 gm. N).
	5		60	2.192	115	420	
	6		54	1.888	110	380	
19	3		80	2.920	16	460	
	4	12.9	76	3.032	8	490	
	5		172	4.600	0	540	Injected 110 cc. solution of alanine (1.87 gm. N).
	6		80	2.520	27	340	
	7		90	2.620	3	370	
20	3		82	2.950	15	775	
	4	10.5	72	2.760	24	550	
	5		114	3.720	14	600	Injected 65 cc. rapidly, biuret-free casein diges- tion product (0.665 gm. N).
	6		86	2.910	28	700	
	7		100	3.150			
21	4		99	2.850	135	638	
	5	7.1	96	2.820	193	812	
	6		104	3.000	115	537	Injected 80 cc. of mixture of amino-acids (biuret- free casein digest, 0.489 gm. N).
	7		93	2.475	90	425	

TABLE II—*Continued.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Creatine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
22	2		148	3.704	42	730	
	3	16.8	100	3.424	0	720	
	4		130	3.160	87	720	Injected 0.5 mg. of histamine per kilo.
	5	16.22	84	3.000	12	600	
	6		114	2.880	25	740	Injected 1 mg. of histamine per kilo.
	7		92	2.736	0	420	
39	3		65	1.680	69	340	
	4	6.7	55	1.560	40	280	
	5		345	1.320	78	425	Injected 1 mg. of histamine per kilo.
	6		94	1.440	82	180	
27	3		90	1.995	76	400	
	4	6.7	80	1.860	73	308	
	5		440	2.250	107	400	Injected 15 cc. of dog muscle autolysate (0.432 gm. N). Coagulable proteins absent. Proteose trace.
	6		124	1.800	90	175	
28	4	10.5	205	2.580	25	440	
	5		615	4.850	258	975	Injected 70 cc. of dog muscle autolysate (1.95 gm. N). Coagulable proteins absent. Proteose trace.
	6		253	2.780	178	320	

teose, was extremely destructive of tissue (Dog 24, Chart 3 and Table III). Hence, if any particular amino-acid is the toxic agent it had opportunity to assert itself. We may conclude therefore that the amino-acids themselves are incapable of producing destruction of tissue and are not contributing factors in this respect to the toxicity of proteoses.

Recently Dale and coworkers (8) demonstrated the similarity of histamine shock and peptone shock, and Abel and Kubota (9)

claimed that histamine is the responsible agent in the toxicity of Witte peptone. Does histamine, one of the most toxic of the amines, have any influence on tissue catabolism? In Dogs 22

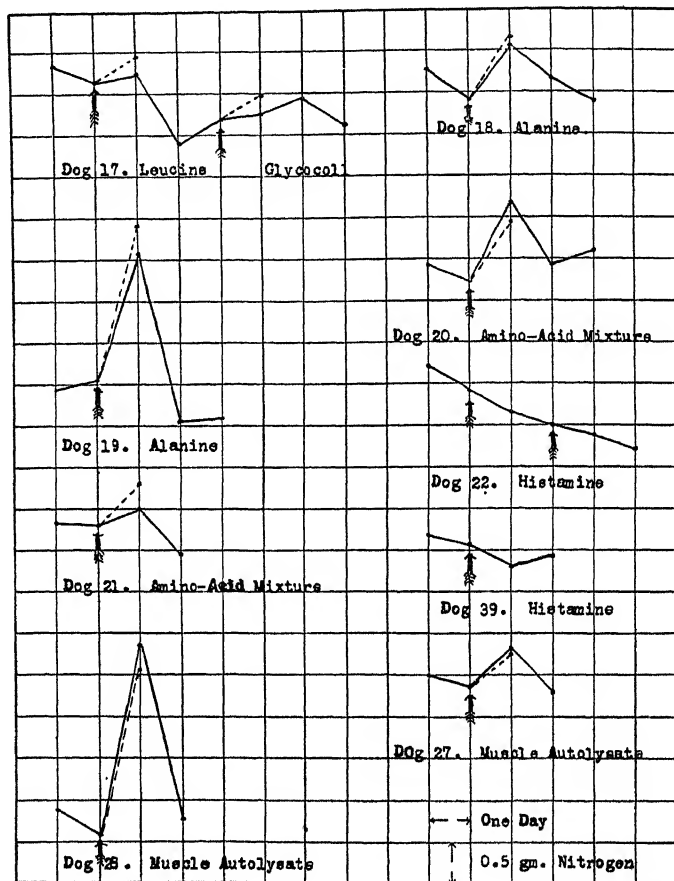


CHART 2. Influence of amino-acids and amines on the daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

and 39 we injected histamine in doses sufficiently large to produce shock and yet the effect on the elimination of nitrogen, creatine, and phosphates was negative. These doses (1 mg. of base per kilo) constitute about 300 times the amount of histamine contained

in a toxic dose of Witte peptone (Dog 5, Chart 3), according to the analysis of Hanke and Koessler (10). Hence the amine content of proteoses as represented by histamine cannot be the catabolic agent of proteoses. This agrees with the findings of Hanke and Koessler (10) who showed that histamine-free proteose gives the typical clinical toxic picture.

Dogs 27 and 28 show that autolysate free from coagulable protein, rich in amino-acids but poor in proteose and peptone as shown by a faint biuret reaction, produced no increase in urinary nitrogen. This substance was prepared by autolyzing 1 kilo of dog's muscle under toluene, for a month, precipitating the proteins, and concentrating the residue. Unfortunately the creatine and phosphates were not determined in the injected material. That the undoubtedly rich content of creatine and phosphates of the injected material would account for the large output of those constituents in Dog 28 which received the undiluted residue, seems to us more rational than to accept the indication that tissue is destroyed. This view agrees with the negative influence on the total nitrogen.

These last two experiments are of interest in indicating that autolysis *in vitro* and autolysis *in vivo* although probably the result of the same mechanism are capable of contrary effects. The autolysis that obtains during septic or aseptic suppuration is toxic, for these are usually accompanied by fever, depression, and as Whipple has shown by tissue catabolism. The explanation it seems to us is to be sought in the general quantitative character of the end-products which the conditions of the autolysis determine, rather than in the production of special products endowed with specific properties. Had the autolysate given to Dogs 27 and 28 contained a sufficient amount of the more complex protein derivatives like proteose, in other words had the digestion been less complete, it would undoubtedly have been toxic also as will be clear from the next series of experiments.

#### *The Influence of Ordinary Proteoses on Catabolism.*

As has been already stated, the question of prime importance is: Are the reactions called forth by Whipple's toxic proteose specific, or are they common to other proteoses? Clinically

the question has been answered. All, with the possible exception of gelatoses, are active. It remains to be seen whether other proteoses induce tissue catabolism.

TABLE III.  
*Influence of Ordinary Proteoses.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
5	2	15.0	110	3.480	112	805	Injected 0.4 gm. per kilo of Witte peptone (0.6 gm. N).
	3		102	3.370	138	717	
	4		455	5.040	292	1,383	
	5		182	5.900	205	997	
6	3	10.3	90	2.325	91	550	Injected slowly 50 cc. of Witte peptone—0.2 gm. per kilo (0.325 gm. N).
	4		76	2.393	108	450	
	5		143	3.060	182	650	
	6		192	3.375	280	450	
	7		106	2.790	69	475	
	8		90	2.400	71	440	
	9		60	2.115			
4a	2	10.2	170	2.610	62	425	Injected 100 cc. of Witte peptone solution—0.3 gm. per kilo (0.59 gm. N).
	3		140	2.784	18	330	
	4		330	3.384	20	720	
	5		220	4.337	133	460	
	6		190	3.469	19	495	
	7		154	3.050	0	470	
4b	3	9.3	86	3.252	23	680	Injected rapidly 90 cc. of Witte peptone for immunity—0.3 gm. per kilo (0.592 gm. N).
	4		106	3.192	37	610	
	5		225	4.755	180	1,295	
	6		148	5.835	350	700	
	7		122	4.965	130	800	

TABLE III—*Continued.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm</i>	<i>mg.</i>	<i>mg.</i>	
23	4	11.6	120	3.020	117	587	Injected pure deuteroproteose egg albumin—0.15 gm. per kilo (0.174 gm. N).
	5		113	2.640	84	650	
	6		150	3.690	149	1,050	
	7		135	2.650	54	525	
24	4	16.3	140	3.750	135	725	Injected pure deuterocaseose—0.3 gm. per kilo (0.370 gm. N).
	5		120	3.160	60	675	
	6		365	4.710	320	1,150	
	7		390	5.730	529	800	
	8		188	3.130	31	675	
25	4	6.8	52	1.345	9	180	Injected deuteroproteose egg albumin—0.15 gm. per kilo (0.100 gm. N).
	5		40	1.470	43	307	
	6			1.430	57	450	
	7		102	2.420	41	330	
	8		90	1.300	22	230	
30	4	17.3	130	4.620	31	625	Injected 0.1 gm. per kilo Vaughan's crude soluble poison in 50 cc. of saline solution (0.167 gm. N).
	5		170	5.025	50	775	
	6		260	6.660	173	2,400	
	7		900	9.850	655	1,670	
	8		100	14.700	490	1,700	

In Table III and Chart 3 are recorded experiments along this line. Dog 5 received a large dose of Witte peptone and it will be observed in the next 2 days he excreted about 2.5 gm. of extra nitrogen. The creatine was more than doubled and the P<sub>2</sub>O<sub>5</sub> almost doubled. The significance of an increased nitrogen output has already been discussed by Whipple (5,6) and is a clear argument for tissue destruction. The marked rise in creatine is

an added indication of the same, as has long been known. During fasting, the involution of the muscular uterus, muscular atrophy,

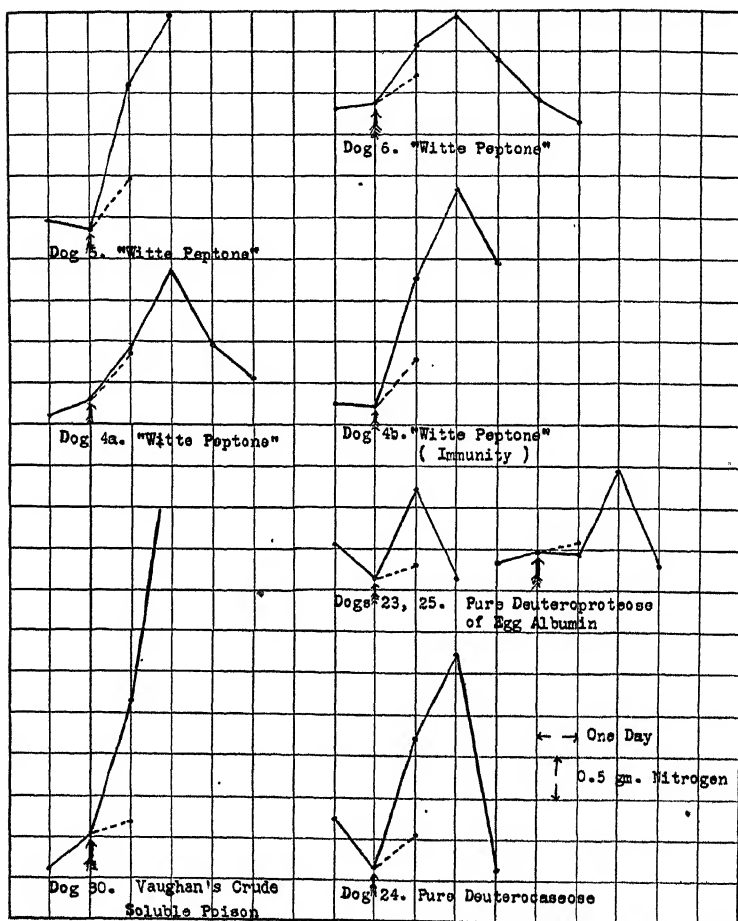


CHART 3. Influence of proteoses on daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

or wherever else muscle tissue is disintegrated extra creatine appears in the urine. But more than a confirmation of the evidence that the extra nitrogen gives, the large creatine output

points to the seat of the catabolism, a question that Whipple raised but could not answer from his experiments; namely, the muscle tissue.

The large output of extra phosphorus is an added indication of tissue catabolism and probably of muscle in particular. Forbes and Keith (11) in a review of the literature on this subject bring out the facts that the destruction of phosphorus-containing proteins as in general catabolism, violent exercise, the destruction of leucocytes, cancer, tuberculosis, septic conditions, fever, and acidosis, results in an excess output of phosphates. In peptone shock we have an acidosis but in these dogs shock was avoided by slow injection, hence it must be the tissue destruction factor which accounts for the extra phosphate. Since the other conditions mentioned are largely muscle wasters, the extra phosphate output here may be considered an index of muscular catabolism.

Dog 6 received half the dose of Witte peptone of Dog 5. The response in the three directions indicated is not as marked but it is definite.

Dog 4a received a dose intermediate between Dogs 5 and 6 and the response was intermediate by all three criteria. We may say that the size of the dose is an important factor in the severity of the catabolism induced. This has long been known with regard to the clinical effects, small doses being innocuous and larger doses fatal.

There is no way of accurately comparing the degree of response of our dogs with Whipple's because this author does not state his dosage in terms of nitrogen so that judgment can be made of the amount of proteose given. On the whole, our effects are neither as great nor as prolonged, but certainly of the same kind.

Dog 4b received the same dose a second time several weeks later to test for immunity but the response was much greater the second time. This may have been due to the rapidity of the injection which we know from the clinical reactions is an important factor. All three criteria of tissue catabolism are strongly present.

In order to controvert the idea that Witte peptone is an impure proteose and therefore not a fair criterion of what other proteoses will do, to Dogs 23, 24, and 25 we gave pure proteoses prepared from egg white and casein.<sup>3</sup> The response of Dogs 23 and 24

<sup>3</sup> The material formed from egg white and casein was prepared by pepsin digestion then saturated with ammonium sulfate after neutralization



although characteristic was not marked. This can be accounted for by the small dose, 0.15 gm. per kilo, which was necessitated by the fact that larger doses killed several dogs from the acute effects (blood pressure depression, etc). Dog 24 received 0.3 gm. per kilo at the usual rate and the tissue catabolism was as great as with Witte peptone. Hence the purity or impurity is of small moment so long as there is sufficient proteose present.

It will be observed in our dogs that the excess nitrogen excretion is partially delayed to the 2nd day as with Whipple's dogs. That is to say, the crest of the excretion comes on the 2nd day in most of the cases. Another point of identity is the diuresis, although that is frequently not marked.

As evidence of how much more toxic than ordinary proteoses, other protein split products can be, there is the enormous catabolic influence of Vaughan's crude soluble poison. Dog 30 received only 0.1 gm. per kilo. Witte peptone would have been harmless in that dosage. On the 2nd day the nitrogen output was doubled and on the 3rd day trebled. The creatine and phosphate kept pace. What are we to say of the relative toxicity of proteoses? It is true Vaughan's crude soluble poison is not strictly a proteose but it is not far removed from one. It gives the clinical reactions of proteoses, but more intensely. It is partially precipitated by saturation with ammonium sulfate, both filtrate and precipitate being active. It is nearer the protein end of the hydrolytic chain. But proteins as we shall see later although catabolic agents are not nearly as destructive as this substance or even Whipple's toxic proteoses. So that it cannot be the position in the hydrolytic scale that is the whole story. It seems to us reasonable to conclude that probably all true proteoses are catabolic agents, although there is considerable variability in the degree of injury induced.

of the digestion mixture and removal of undigested residue and neutralization precipitate. The proteoses were dissolved in  $H_2O$  and dialyzed free from  $(NH_4)_2SO_4$ . The mixture was filtered from the small precipitate of heteroproteose and the filtrate evaporated and saturated with NaCl which precipitated the so called protoproteose. Treatment of the filtrate from the NaCl saturation with acetic acid precipitated a mixture of proto- and deutero-proteose. The filtrate from the acetic treatment was dialyzed free from NaCl and constituted the yield of deutero-proteose. The solution freed from NaCl was concentrated to small volume and treated with alcohol. The precipitate of deutero-proteoses was washed with boiling alcohol and treated with ether while hot, and ground to a fine white powder.

TABLE IV.  
*Influence of Proteins.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
7	2	8.95	75	2.533	42	360	Injected 150 cc. of solution of crystallized edestin (0.234 gm. N).
	3		60	2.493	32	370	
	4		110	3.753	35	714	
	5		95	3.466	68	290	
	6		53	3.106	51	440	
	7		62	3.080	55	320	
	8		50	2.640	36	290	
10	6	10.9	100	2.760		588	Injected 40 cc. of excelsin solution (0.060 gm. N).
	7		102	2.640	78	488	
	8		200	2.925	121	662	
	9		130	2.850	152	413	
	10		92	2.520		425	
8	2	16.1	154	4.180	81	671	Injected 35 cc. of solution of egg albumin (0.480 gm. N).
	3		112	3.972	79	668	
	4		110	4.060	70	680	
	5		187	4.480	118	998	
	6		130	4.836	81	560	
	7	14.9	108	4.040	48	482	Injected 47.7 cc. of solu- tion of egg albumin (0.411 gm. N).
	8		94	3.920	40	540	
	9		115	4.260	70	720	
	10		110	5.000	42	400	
	11		130	4.146	24	680	
9	4	6.7	100	1.835	73	325	Injected 50 cc. of egg al- bumin solution (0.480 gm. N).
	5		86	1.845	108	362	
	6		148	2.344	125	700	
	7		130	4.590	323	500	
			130	3.375	177		

TABLE IV—*Continued.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg</i>	<i>mg.</i>	
11	2	16.8	104	3.980	67	700	Injected 110 cc. of gelatin solution (0.498 gm. N).
	3		125	3.440	58	780	
	4		130	3.008	0	680	
	5		400	3.320	1	925	
	6		120	3.048	3	580	
	7		90	2.912	9	480	
12	3	11.10	68	2.448	0	480	Injected rapidly 94 cc. of gelatin solution (0.580 gm. N).
	4		84	2.916	11	580	
	5		110	3.030		665	
	6		90	3.156	20	454	
	7		88	2.920		520	
13	4	7.2	90	3.030	80	575	Injected 80 cc. of gelatin solution (0.407 gm. N).
	5		120	3.360	57	700	
	6		110	3.015	46	388	
	7		95	2.580	9	375	

*Influence of Foreign Proteins.*

Although it has long been known that foreign proteins intravenously introduced produce an increased nitrogen output (12), fever (2), and other toxic symptoms, it seemed wise to repeat this type of experimentation in order to compare the degree of catabolic effect with the similar effect of protein split products. Accordingly Table IV and Chart 4 contain the records of a few experiments.

Dog 7 received a dilute solution of edestin. The influence on the nitrogen and phosphates was quite marked, but on creatine only slight. Dog 10 responded with slight extra output of nitrogen and phosphates and a marked extra output of creatine. In view of the very small dose the effect was decidedly positive. Dogs 8 and 9 received egg albumin solution. The effect is in all essentials indicative of a tissue destruction.

Dogs 11, 12, and 13, aside from the slight effect on phosphates which cannot be stressed in itself, responded indifferently to large doses of gelatin solution. This failure to behave like other proteins is not contradictory to the general influence of proteins.

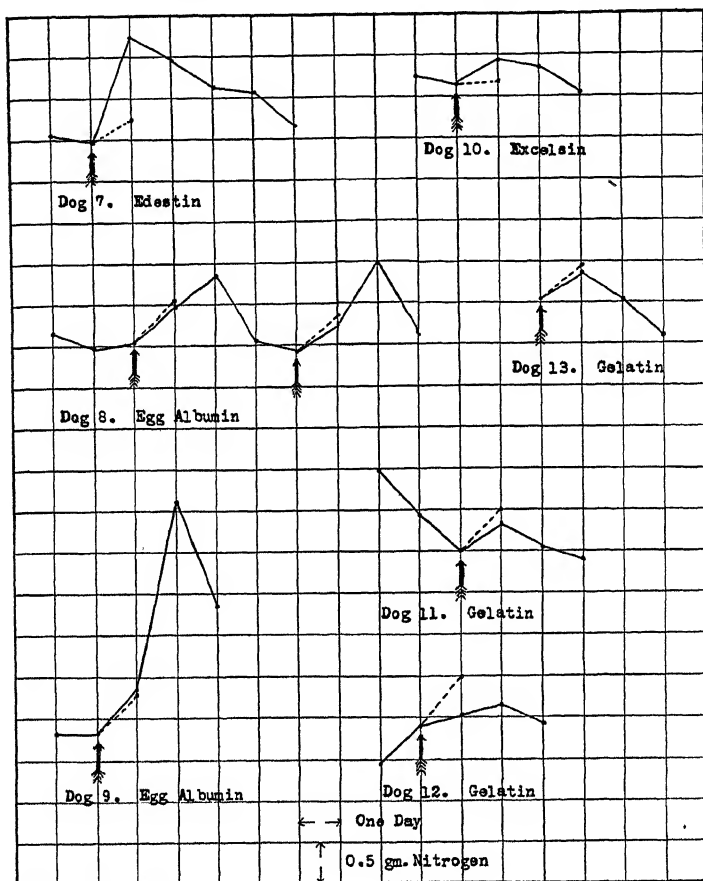


CHART 4. Influence of proteins on daily excretion of nitrogen. Arrows show time of injection. Dotted lines show amount of nitrogen injected.

Gelatin is not anaphylactogenic (13), its gelatoses are innocuous, it is deficient in important amino-acids. It confirms, however, our view that the colloidal nature of the solution is of small moment in the effects under consideration.

On the whole although the proteins are decided catabolic agents they are so to no greater degree than the proteoses. Friedmann and Isaac have shown that even homologous sera are toxic in this respect. Vaughan suggests that this is due to the fact that the proteins have become "foreign" in the process of coagulation. The same may be held for the injury to body protein during an inflammatory process. Injured protein is foreign protein and hence is a catabolic agent. In explaining then, the cause of tissue catabolism during inflammation, the attention must not be directed to the products of the injured proteins alone, but to the parent proteins as well.

#### DISCUSSION.

We believe we have answered the first question we set out to answer, to wit, that the catabolic reactions called forth by the toxic proteose of Whipple and his coworkers, are not specific but are common to this genus of compound, although to a variable degree. Whether there are other products capable of the same effect, we have already pointed to proteins themselves, and to Vaughan's crude soluble poison. We may add in anticipation of the following paper, in which the subject is given in detail, that certain nucleic acids are also capable of this catabolic effect to a very marked degree.

#### SUMMARY.

1. Ordinary proteoses induce catabolism of tissue in fasting dogs, as evidenced by a large output of urinary nitrogen, creatine, and phosphates.
2. Both pure and impure proteoses are effective.
3. Proteoses differ in the degree of their effect. The dosage and rate of injection are factors.
4. Proteins except gelatin are also capable of this effect.
5. The amino-acids, histamine, and *in vitro* autolysates, are without influence.

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# **STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.**

## **VIII. THE INFLUENCE OF NUCLEIC ACIDS ON THE METABOLISM OF FASTING DOGS.**

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Whipple and Cooke<sup>1</sup> have demonstrated that the toxic proteose recovered from obstructed intestines or isolated loops, induces a marked destruction of tissue upon injection into normal dogs, as evidenced by the large output of urinary nitrogen. In the preceding paper we have shown that other proteoses possess the same property. In view of the marked similarity in physiological behavior between proteose and nucleic acid, with regard to the blood pressure depression, delayed coagulability, increased lymph flow, etc., as described by Mendel, Underhill, and White,<sup>2</sup> it seemed opportune to study whether this resemblance can be extended to the catabolic effect under discussion.

Accordingly pursuing the same method of investigation as recorded in the preceding paper we gave fasting dogs, with a constant nitrogen output, intravenous injections of representative animal and plant nucleic acids and noted the effect on the urinary nitrogen, creatine, and phosphates. The animal nucleic acids were prepared from thymus of the calf, spleen of the steer, and spleen and pancreas of the dog, by the methods described by Jones<sup>3</sup> and Baumann.<sup>4</sup> The vegetable nucleic acid was prepared

<sup>1</sup> Whipple, G. H., and Cooke, J. V., *J. Exp. Med.*, 1917, xxv, 461.

<sup>2</sup> Mendel, L. B., Underhill, F. P., and White, B., *Am. J. Physiol.*, 1902-03, viii, 377.

<sup>3</sup> Jones, W., *Nucleic acids. Their Chemical properties and physiological conduct*, New York and London, 2nd edition, 1920, 103.

<sup>4</sup> Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 118.



from fresh brewers' yeast by the method of Baumann.<sup>5,6</sup> The substances were dissolved in normal saline solution and neutralized before injection.

*Effect of Plant Nucleic Acid.*

In Chart 1 and Table I are recorded the results of experiments with yeast nucleic acid.

Dog 32 received a standard dose slowly. The output of nitrogen, creatine, and phosphates was considerably increased over the basal level. Dog 33 received the same dose rapidly and responded with a much greater output of each of these constituents. As is the case with the injection of proteose the rate of injection is a factor of importance.

Dog 31 received a smaller dose than any of the others but the output of nitrogen and creatine was very much greater. This animal was very badly shocked. As is apparent from the experiments of Mendel, Underhill, and White<sup>2</sup> there is a considerable individual variation in response. The effect on Dogs 40 and 41 was characteristic.

The nitrogen output in all of these animals increased. It varied from 1 to 4 gm. in excess of the basal level, on the first day of the injection. The excretion continued above normal for several days thereafter (Chart 1). This is a clear argument for a significant tissue destruction.

The effect on the creatine output is a striking confirmation of the tissue destruction which the large nitrogen excretion indicates. It, moreover, points to the muscular tissue as the seat of this effect.

The evidence of the phosphates is not at once apparent, for the output above the amount injected does not seem large—no more than a few hundred mg. But the true condition is really obscured. Consideration of the protocols of the succeeding experiments on animal nucleic acids will show that the results are entirely negative as far as the effect on tissue destruction is concerned. We may consider those experiments from our view-

<sup>5</sup> Baumann, E. J., *J. Biol. Chem.*, 1918, xxxiii, p. xiv.

<sup>6</sup> Dr. Emil J. Baumann was kind enough to send us samples of spleen and yeast nucleic acids.

point, as controls. There, it will be observed, the phosphorus injected is not entirely eliminated in the urine. Not more than half the amount given is excreted. Hence, since in the experi-

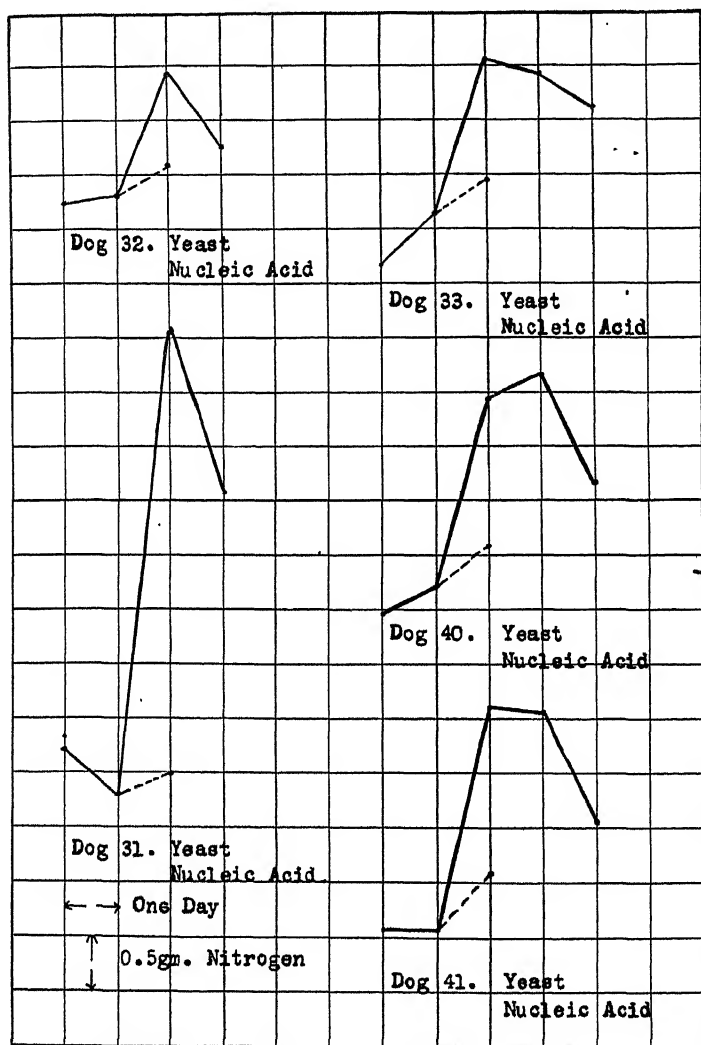


CHART 1. Influence of plant nucleic acid on the daily excretion of nitrogen. Dotted lines show amount of nitrogen injected.

TABLE I.  
*Influence of Plant Nucleic Acid.*

Dog.	Day.	Weight.	Urine vol- ume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		kg.	cc.	gm.	mg.	mg.	
32	3		122	3.225	172	1,000	
	4	13.4	130	3.315	70	990	
	5		350	4.410	153	1,600	Injected slowly 125 cc. of yeast nucleic acid, 2 per cent solution (0.300 gm. N, 750 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		134	3.765	126	680	
33	3		104	3.180	52	887	
	4	13.04	92	3.645	42	962	
	5		340	5.040	240	2,000	Injected rapidly 116 cc. of yeast nucleic acid, 2 per cent solution (0.302 gm. N, 760 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		130	4.950	240	725	
	7		110	4.635	135	800	
31	4		92	2.235	132	413	
	5	11.00	96	1.825	194	507	
	6		200	6.050	237	1,250	Injected 50 cc. of 2 per cent yeast nucleic acid (0.120 gm. N, 320 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		188	4.550	462	500	
40	3		80	1.970	118	530	
	4	8.00	100	2.210	115	505	
	5		700	3.950	239	1,560	Injected 0.5 gm. per kilo of yeast nucleic acid (0.390 gm. N, 970 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		150	4.170	307	530	
	7		90	3.160			
41	3		125	3.590	138	613	
	4	12.7	100	3.590	65	760	
	5		685	5.150	200	1,790	Injected 0.5 gm. per kilo of yeast nucleic acid (0.540 gm. N, 1,350 mg. of P <sub>2</sub> O <sub>5</sub> ).
	6		210	5.100	380	810	
	7		160	4.040			

ments under discussion the output is not only as great as the amount injected but greater, we may conclude that there was a considerable excretion of phosphates above the basal level. This confirms the evidence derived from the consideration of the effect on the total nitrogen and the creatine.

It may be concluded, therefore, on grounds similar to those taken in the preceding paper that plant nucleic acid induces a marked tissue destruction when introduced directly into the circulation.

### *Effect of Animal Nucleic Acids.*

Contrary to our expectations we were unable to demonstrate a similar effect as a result of the injection of animal nucleic acids. In Table II and Chart 2 are recorded the results of these experiments.

In the five experiments where pure preparations from various sources were used, the results were uniformly negative with regard to the output of nitrogen. The amount injected was excreted, no more. Dog 37 showed a slight rise, this result, however, could not be repeated in Dog 38. The phosphate output to which we have already alluded, is in conformity with this finding. In Dogs 34 and 35 there were significant increases in the output of creatine. It is difficult to interpret these findings standing alone, unsupported by evidence from the nitrogen and phosphate output. We are inclined to disregard them, especially in view of the fact that this increase did not occur uniformly.

It may therefore be concluded that animal nucleic acid injected directly into the circulation unlike plant nucleic acid gives no evidence of inducing tissue destruction. In short, it is not a toxic substance.

### DISCUSSION.

This investigation, as was stated in the preceding paper, was undertaken primarily in order to find substances other than proteoses that are capable of stimulating catabolism and that might be formed as a result of tissue injury. We have demonstrated that plant nucleic acid is such a substance. It is reasonable to believe in view of the fact that the bacterial cell is largely composed of nucleoprotein, that the nucleic acids resulting from the

TABLE II.

*Influence of Animal Nucleic Acids.*

Dog.	Day.	Weight.	Urine volume.	Total N.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	Remarks.
		<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
34	4		110	1.800	175	463	
	5	10.5	110	1.945	120	463	
	6			2.190	235	960	Injected 0.3 gm. per kilo of thymus nucleic acid in 50 cc. (0.369 gm. N, 920 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		120	1.920	87	410	
35	4		105	3.240	20	600	
	5	11.4	110	3.150	42	600	
	6		90	2.955	100	835	Injected 50 cc. of thymus nucleic acid, 0.25 gm. per kilo (0.360 gm. N, 900 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		109	3.000	51	538	
36	4		97	3.340	43	613	
	5	11.5	116	3.250	10	535	
	6		190	3.602	79	755	Injected 0.1 gm. per kilo of dog nucleic acid (pan- creas and spleen, 0.158 gm. N, 400 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		170	3.450	109	535	
	8		115	2.860	30		
37	4		110	2.800	88	710	
	5	17.0	135	2.940	102	663	
	6		224	3.550	96	1,065	Injected 3 per cent solu- tion of spleen nucleic acid (0.320 gm. N, 800 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		137	3.420		325	
	8		115	2.820	76	362	
38	4		132	3.190	96	638	
	5	15.0	114	2.920	67	675	
	6		400	3.450	69	900	Injected 0.4 gm. per kilo of spleen nucleic acid (0.562 gm. N, 1,400 mg. of P <sub>2</sub> O <sub>5</sub> ).
	7		135	2.880	150	650	

decomposition of this substance might, in septic processes, be a contributing agent in the general intoxication. However, this source of nucleic acid is conceivably not great. The host's own nucleoproteins would be a much richer source, and as Whipple and Cooke and their coworkers have shown for proteoses, the destruction of the host's tissues is the real source of the intoxicat-

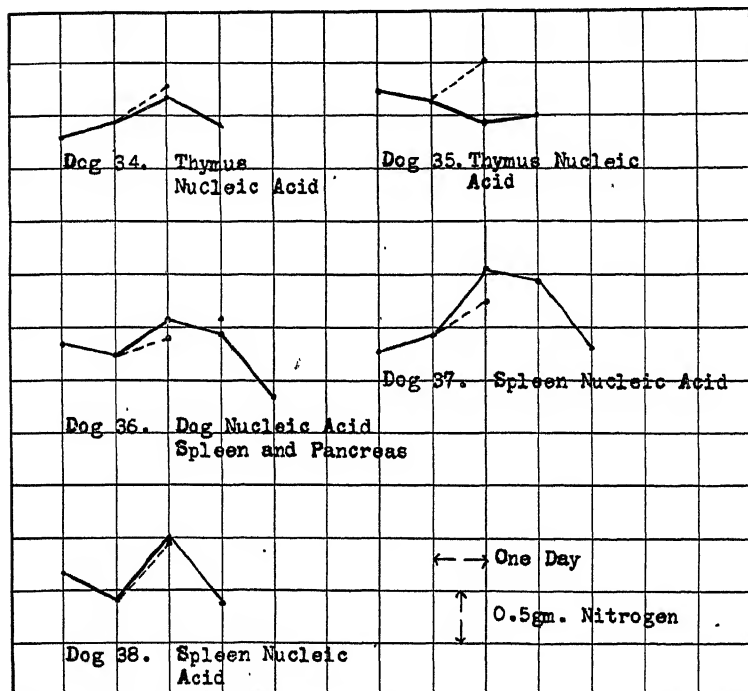


CHART 2. Influence of animal nucleic acids on the daily excretion of nitrogen. Dotted lines show the amount of nitrogen injected.

ing substance. Since we have demonstrated that animal nucleic acid is not toxic it is probable that this type of compound is not an important factor in the intoxication of inflammatory processes. Moreover, bacterial nucleic acid has been little studied and it is not clear whether it would behave like the animal or plant variety. Hence, little can be definitely argued for the rôle of nucleic acids, in inflammatory intoxications.

But our finding with regard to the failure of animal nucleic acid to injure tissue, opens the question of whether this compound resembles proteoses in physiological behavior at all. Mendel, Underhill, and White<sup>2</sup> showed that the plant nucleic acid they used, like proteose, caused a fall in pressure, delayed coagulability of the blood, increased flow of lymph, etc. We have recently

TABLE III.  
*Acute Effects of Plant and Animal Nucleic Acids.*

Dog.	Time.	Blood pressure.	Clotting time.	Hemoglobin.	Alkali reserve CO <sub>2</sub> volume.	Remarks.
	<i>min.</i>	<i>mm. Hg</i>		<i>per cent</i>	<i>per cent</i>	
30	0	130	3 min.	100	65	Injected spleen nucleic acid, 0.3 gm. per kilo in normal saline solution.
	1					
	2	60				
	3	130	3 hrs.	92	65	
	63	130		93		
	300	130	45 min.	100	59	
32	0	97	12 min.	100	58	Injected spleen nucleic acid, 0.5 gm. per kilo.
	1					
	2	30	3 hrs.	92	50	
	7	60				
	12	80				
	27	112		97	55	
	180	115	50 min.	101	53	
21	0	108	4 min.	100	41	Injected yeast nucleic acid, 0.3 gm. per kilo.
	1					
	2	10	11 min.	86		
	12	15		92	28	
	32	55	5 min.	96	37	
	92	22		115	20	
	152	10		128	15	

been able to add a concentrated blood<sup>7</sup> as measured by the increase in hemoglobin, and an acidosis<sup>7</sup> as measured by the alkali reserve.

On repeating this work with pure animal nucleic acid we found that the effect on the blood pressure was exceedingly fleeting and not very profound, that in some cases the coagulability was markedly delayed but that there was no effect on the blood concentra-

<sup>7</sup> Authors' unpublished data.

tion (hence probably none on the lymph flow) and no effect on the alkali reserve. Table III gives three typical protocols, two of animal nucleic acid and the other of plant nucleic acid for comparison. We used large doses and concentrated solutions of spleen nucleic acid neutralized before injection. Hence it must be concluded that animal nucleic acid differs also from plant nucleic acid in not showing the typical peptone type of shock. This finding is at variance with the work of Bang<sup>8</sup> who worked with guanylic acid of the pancreas.

#### SUMMARY.

1. Yeast nucleic acid induces a destruction of tissue, when introduced directly into the circulation, as evidenced by an increased output of nitrogen, creatine, and phosphates.

2. Animal nucleic acid has no such effect.

3. Animal nucleic acid differs also from plant nucleic acid in failing to give typical peptone type of shock.

4. Nucleic acids probably play a small rôle in the intoxication of inflammatory processes.

<sup>8</sup> Bang, I., quoted by Kossel, A., *Z. physiol. Chem.*, 1900-01, xxxi, 410; 1901, xxxii, 201.





## STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

### IX. ALKALI RESERVE AND EXPERIMENTAL SHOCK.

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New Haven.)*

(Received for publication, August 1, 1921.)

The relation of acidosis to shock has been the subject of numerous investigations leading to a diversity of views. That a diminished alkali reserve may occur in shock no one will deny. On the other hand, evidence of a depleted alkali reserve in shock may be lacking. From a survey of all the evidence one is forced to the conclusion that the condition of acidosis in shock should be viewed from the aspect of an accompanying factor rather than as a contributing cause to the condition. It should be emphasized, however, that with shock once established an accompanying acidosis may directly contribute to a fatal outcome.

An investigation of shock induced by various protein derivatives has afforded us the opportunity of studying the relation of diminished alkali reserve to the shock-like condition evoked.

#### *Methods.*

The experiments were carried out upon full grown, well nourished dogs that were allowed to fast for a period of 24 hours previous to the experiment. Anesthesia was produced by a mixture of morphine sulfate (0.01 gm. per kilo), atropine sulfate (0.001 gm. per kilo), and ether. Blood pressure was recorded with a mercury manometer attached to the right carotid artery. Injections were made into a jugular vein. Unless otherwise specified all injections were made rapidly—within a minute—and the volume of fluid introduced did not exceed 50 cc. Blood

for analysis was drawn from a femoral artery and alkali reserve was determined by the method of Van Slyke. Witte peptone, purified proteoses, Vaughan's crude soluble poison, and nucleic acid were injected in doses of 0.3 to 0.5 gm. per kilo. Histamine dosage varied from 1 to 2 mg. per kilo calculated as the base.

*The Relation of Alkali Reserve to Shock Produced by Various Protein Derivatives.*

As may be observed from the table a shock-like condition as measured by low blood pressure was induced by the intravenous injection either of Witte's peptone, Vaughan's crude soluble poison, purified proteoses from casein, nucleic acid, or histamine hydrochloride. The data are arranged to emphasize the relation of the height of blood pressure and duration of low pressure to the alkali reserve. Thus, the word "maintained" in the table indicates a continued very low pressure, varying from 10 to 30 mm. of mercury. The word "temporary" designates a fall of pressure perhaps even to a very low point but with either a progressive or rapid rise to near normal limits. In general it may be noted that in these experiments, especially with Witte peptone, where low pressure has been maintained for a significant interval the alkali reserve shows the greatest decline. This, however, is by no means invariable. Generally, also, even though the fall in pressure is only temporary there is some indication of a decrease in alkali reserve. A striking difference is shown between the influence of yeast nucleic<sup>1</sup> acid and that of animal origin. In other respects the same fundamental difference in the behavior of these two types of nucleic acid when introduced into the body has been observed.<sup>2</sup> Less influence upon alkali reserve is to be seen from the injection of histamine than from that of any other substances introduced.

<sup>1</sup> Kindly furnished by Dr. E. J. Baumann.

<sup>2</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, xlviii, 523.

*The Relation of Low Blood Pressure to Alkali Reserve.*

Experiment.	Low blood pressure.	Alkali reserve (volumes per cent).		Fate.
		Normal.	Later.	
Witte peptone.				
2	Maintained.	67	25	Died.
4	"	59	43	"
5	"	57	15	"
6	"	58	24	"
9	"	68	30	"
45	"	44	22	"
3	Temporary.	63	58	
17	"	46	37	
43	"	53	42	
47	"	51	33	
Deuteroproteoses (casein).				
33	Temporary.	54	34	
Vaughan's crude soluble poison (casein).				
19	Maintained.	50	27	
24	"	43	32	
36	"	50	46	
18	Temporary.	48	33	
35	"	61	34	
20	"	52	36	
38	"	59	43	
Nucleic acid (yeast).				
21	Maintained.	41	15	
Nucleic acid (thymus).				
32	Temporary.	58	53	
30	"	65	59	
29	"	55	54	
15	"	53	49	
28	"	50	35	
Histamine (hydrochloride).				
8	Maintained.	59	32	
11	"	58	42	
13	"	51	60	
14	"	50	50	

Why is it that there is such a diversity of response in the experiments presented? Undoubtedly in all the observations cited, with the possible exception of thymus nucleic acid, a shock-like condition intervened. This shock-like condition is accompanied by disturbances in respiration and circulation which may give rise to the production of acid products. Sufficient production of acid substances in the time interval possible under the experimental conditions hardly seems an adequate explanation. It seems to the authors that a much more plausible explanation lies in a decrease in the capacity of the body to excrete acid whether formed at a normal or at an accelerated rate. Thus a characteristic of low pressure is the appearance of a condition of anuria with a consequent greatly diminished ability to eliminate acid. We would suggest therefore that one large factor for the decrease in alkali reserve in the observations submitted is the failure of the renal mechanism incident to the low pressure. Inspection of the data from this view-point reveals a fairly close correlation between maintained low pressure and decreased alkali reserve. An apparent lack of correlation is encountered with the low pressure induced by histamine. An analysis of the details of the blood pressure fluctuations demonstrates that the pressure in the histamine experiments could not be maintained below 30 mm. of Hg even though histamine were continually introduced. In many of the experiments with other substances employed especially Witte peptone, a lower pressure obtained. Cushny<sup>3</sup> states that urine may continue to be secreted at 30 to 40 mm. of Hg. We would therefore suggest that the fall of alkali reserve noted in these experimental conditions of shock is directly related to failure of the renal mechanism to excrete acid—this inability of the kidney being induced by a maintained low blood pressure. Further work in this direction is in progress.

*Addendum.*—Since the above was written a paper has appeared by Eggstein (Eggstein, *J. Lab. and Clin. Med.*, 1921, vi, 481) on alkali reserve and protein shock. Our own experiments in as far as they are comparable yield results in many respects in confirmation of those of Eggstein.

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<sup>3</sup> Cushny, A. R., *The secretion of the urine*, New York and London, 1917.

# STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

## X. THE INFLUENCE OF NUCLEIC ACID ON THE METABOLISM OF THE FASTING RABBIT.\*

By FRANK P. UNDERHILL AND MARY LOUISA LONG.

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New Haven.)

(Received for publication, August 1, 1921.)

In a previous paper<sup>1</sup> in this series it has been pointed out that the introduction of yeast nucleic acid into the circulation of the fasting dog induces a destruction of tissue as evidenced by an increased output of nitrogen, creatine, and phosphates. The acute effects of this nucleic acid resemble strongly those characteristic of peptone shock. Since rabbits are more or less refractory to peptone injection in this respect experiments similar to those for dogs previously reported have been carried through on rabbits with nucleic acid to determine whether the nucleic acid influence as seen in dogs is capable of being extended to the rabbit.

### *Methods.*

The animals were fasted for 2 days previous to the injection. Urinary excretion was divided into 24 hour periods by pressure upon the bladder through the abdominal wall. Total nitrogen determinations were made in duplicate by the Kjeldahl method, preformed creatinine by Folin's method, and total creatinine by the procedure of Benedict. Phosphates were estimated by titration with uranium acetate. Blood drawn from an ear vein was precipitated and analyzed for non-protein nitrogen by the method

\* The data of this paper are taken from the essay by Mary Louisa Long in partial fulfillment of the requirements for the degree of Master of Science, Yale University, 1920.

<sup>1</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, *xlvi*, 523.

of Folin and Wu. Hemoglobin was determined by the procedure of Cohen and Smith. Nucleic acid, containing 12.2 per cent nitrogen and 8.2 per cent phosphorus was prepared from brewers' yeast by the method of Baumann.<sup>2</sup> A fresh 1 per cent solution was used for each injection made by dissolving the nucleic acid in hot 0.9 per cent NaCl solution with the aid of a few drops of concentrated KOH. The injections were made on the basis of nitrogen content of the solution. Amounts varying from 20 to 60 cc. according to the size of the animal were slowly injected into the circulation through the marginal ear vein. The immediate effects of the injection were an accelerated respiration and a transitory shock-like condition.

### *Control Experiments.*

#### *The Influence of Fasting, Etc., upon Nitrogen Excretion in Rabbits.*

To determine whether the increased nitrogen output caused by the injection of nucleic acid really means an increased tissue catabolism, or is due to other factors, such as fasting, rabbits in a fasting condition were injected with a slightly alkaline solution. The following experiments show that under these conditions the nitrogen rises slightly on the 2nd day of fasting, but afterwards keeps on a level or falls. The creatinine remains practically unchanged, but there is a slight rise in creatine. The phosphates show practically no change.

The nitrogen gradually increases, showing a marked rise on the last 2 days of the experiment. This probably accounts for a secondary rise observed in some of the injected rabbits. The creatinine keeps on a level as in the saline injected animals, but the creatine rises very markedly, increasing to eight times its original amount on the last day of fasting. This no doubt indicates a destruction of muscular tissue, and was usually observed in very thin animals. The phosphates remained on a level as before.

<sup>2</sup> Baumann, E. J., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 118.

*The Influence of Intravenous Administration of Nucleic Acid upon Nitrogen Excretion in the Rabbit.*

In testing the influence of the intravenous injection of nucleic acid upon nitrogen excretion, varying amounts were given in order to determine the most effective dose. Rabbits 8 and 9

TABLE I.

*The Influence of Fasting, Etc., upon Nitrogen Output. Control Experiments.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 6. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 8	200	55	0.584	0.050	0.004	0.150	Mar. 9, injection of 67 cc. of faintly alkaline 0.9 per cent NaCl solu- tion.
" 9	90	90	0.714	0.063	0.011	0.204	
" 10	37	105	0.602	0.044	0.010	0.150	
" 11	80	72	0.882	0.065	0.030	0.336	
" 12	80	52	0.884	0.058	0.043	0.226	
Rabbit 22. Body weight 1.85 kilos.							
Apr. 29	40	70	0.562	0.069	0.038	0.202	Apr. 30, injection of 52 cc. of faintly alkaline 0.9 per cent NaCl solu- tion.
" 30	55	60	0.942	0.072	0.018	0.192	
May 1	0	50	0.850	0.099	0.015	0.258	
" 2	0	34	0.872	0.084	0.006	0.202	
" 3	40	23	0.854	0.079	0.011	0.182	
Rabbit 23. Body weight 1.62 kilos.							
May 2	170	75	1.110	0.108	0.013	0.162	May 3, injection of 40 cc. of faintly alkaline 0.9 per cent NaCl solu- tion.
" 3	100	98	0.874	0.069	0.008	0.180	
" 4	80	100	1.035	0.075	?	0.293	
" 5	0	32	0.756	0.065	0.036	0.222	
" 6	0	30	0.765	0.073	0.026	0.190	

were given very small doses of nucleic acid, 0.05 gm. per kilo of body weight with practically no effect, as shown in Table III.

The picture is the same as shown in the control experiments. The fact that the nitrogen rises to a maximum the day after the injection and then returns to normal might indicate a slight destructive action of a small dose.



Table IV shows the effect of increasing the amount of nucleic acid injected. Rabbit 7 received 0.1 gm. per kilo of body weight. The nitrogen rose 0.4 gm., an amount not to be accounted for by

TABLE II.  
*Effect of Plain Fasting on Nitrogen Output.*

Date.	Water intake	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 19. Body weight 1.68 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Apr. 17	0	32	0.474	0.054	0.008	0.356	
" 18	100	30	0.589	0.046	0.008	0.298	
" 19	5	32	0.726	0.052	0.015	0.230	
" 20	85	55	1.056	0.058	0.051	0.258	
" 21	100	130	1.697	0.056	0.074	0.363	

TABLE III.  
*Effect of Injection of 0.05 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Vol-ume.	Total nitro-gen.	Creati-nine.	Crea-tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 8. Body weight 2.7 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 14	0	56	0.938	0.095	0.013	0.186	Mar. 14, injection of a
" 15	80	86	0.977	0.095	0.009	0.247	1 per cent nucleic acid
" 16	0	150	1.045	0.096	0.014	0.278	solution = 0.0216 gm.
" 17	100	82	0.951	0.098	0.020	0.296	of nitrogen.
Rabbit 9. Body weight 2.4 kilos.							
Mar. 25	0	60	0.876	0.062	0.005	0.356	Mar. 26, injection of 15
" 26	60	50	0.969	0.070	0.038	0.284	cc. of a 1 per cent nu-
" 27	60	36	1.072	0.101	0.029	0.220	cleic acid solution =
" 28	100	35	0.933	?	?	0.304	0.0192 gm. of nitrogen.
" 29	70	35	0.920	?	?	0.184	

the small amount of nitrogen injected in the nucleic acid solution. The creatinine, creatine, and phosphates were unaffected by the injection.

In Table V a very marked effect on the urinary nitrogen is shown for Rabbits 3 and 14, after an injection of 0.2 gm. of

TABLE IV.

*Effect of Injection of 0.1 Gm. of Nucleic Acid on Nitrogen Output.*

Date.	Water intake	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 7. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 13	0	48	0.462	0.070	0.015	0.104	Mar. 14, injection of 22.3 cc. of a 1 per cent nucleic acid solution = 0.016 gm. of nitrogen.
" 14	35	44	0.690	0.071	0.010	0.172	
" 15	40	120	1.066	0.081	0.024	0.207	
" 16	0	40	0.774	0.053	0.009	0.167	
" 17	100	76	0.558	0.063	0.028	0.251	

TABLE V.

*Effect of Injection of 0.2 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 3. Body weight 1.9 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Feb. 12	200	13	0.354	0.043	0.005	0.045	Feb. 13, injection of 38 cc. of a 1 per cent nu- cleic acid solution = 0.049 gm. of nitrogen.
" 13	55	46	0.427	0.084	0.005	0.102	
" 14	0	126	1.431	0.093	0.099	0.105	
" 15	100	140	2.196	0.090	0.166	0.390	
" 16	25	90	1.425	0.032	0.155	0.288	
Rabbit 14. Body weight 1.6 kilos.							
Apr. 5	70	32	0.708	0.060	0.019	0.130	Apr. 6, injection of 40 cc. of a 1 per cent nu- cleic acid solution = 0.051 gm. of nitrogen.
" 6	100	60	0.788	0.049	0.011	0.178	
" 7	20	134	1.120	0.034	0.088	0.258	
" 8	100	77	1.359	0.046	0.052	0.318	
" 9	100	100	1.947	0.042	0.105	0.386	

nucleic acid per kilo of body weight. In Rabbit 3 the nitrogen is increased 1 gm. on the day after injection, and rises still higher on the next day. The creatinine is not changed markedly, but

the creatine goes up tremendously, indicating a great tissue destruction. It is interesting to note that the rise in phosphates comes on the 3rd day after injection. Rabbit 14 did not show as striking effects as Rabbit 3. The nitrogen increased 0.5 gm. on the day following injection and continued to rise on the following days. Creatine is quadrupled, and the phosphates are steadily increased; however, this and the high increase in nitrogen on the last 2 days are probably fasting factors.

TABLE VI.

*Effect on Urinary Nitrogen after Injection of 0.3 Gm. of Nucleic Acid per Kilo.*

Date.	Water intake.	Urine.					Remarks.
		Volume.	Total nitrogen.	Creatinine.	Creatine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 5. Body weight 1.76 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 8	200	53	1.184	0.063	0.028	0.140	Mar. 9, injection of 65.5 cc. of a 1 per cent nucleic acid solution = 0.084 gm. of nitrogen.
" 9	105	105	1.475	0.082	0.063	0.283	
" 10	42	168	1.876	0.096	0.086	0.406	
" 11	100	115	1.877	0.053	0.101	0.442	
" 12	100	150	1.246	0.071	0.197	0.487	
Rabbit 4. Body weight 2.4 kilos.							
Feb. 16	15	59	0.636	0.095	0.031	0.096	Feb. 17, injection of 72 cc. of a 1 per cent solution of nucleic acid = 0.086 gm. of nitrogen.
" 17	0	38	0.894	0.102	0.021	0.161	
" 18	30	105	1.189	0.126	0.078	0.160	
" 19	10	56	0.650	0.108	0.000	0.232	
" 20	35	31	0.621	0.092	0.003	0.206	

Rabbits 4 and 5 were given 0.3 gm. of nucleic acid per kilo. There is a decided rise in both the nitrogen and creatine, as shown in Table VI, but the increase is not as marked as with the 0.2 gm. dose, indicating that larger amounts are no more potent.

These experiments show that the intravenous injection of nucleic acid produces a marked rise in the urinary nitrogen and creatine, and that amounts of 0.2 gm. per kilo of body weight give the most striking results.

*The Influence of Intravenous Injection of Nucleic Acid upon the Non-Protein Nitrogen of the Blood.*

Since the results on urinary nitrogen determinations point toward an increased protein catabolism it is of interest to follow the non-protein nitrogen of the blood, to determine whether there is an accumulation of the catabolic products in the blood. To interpret the results correctly, the hemoglobin curve was likewise followed to note whether there was any blood volume change.

TABLE VII.

*Influence of Intravenous Injection of Nucleic Acid on the Hemoglobin of the Blood.*

Date.	Hemoglobin of the blood.		Remarks.
	Time of drawing blood.	Hemoglobin.	
Rabbit 14. Body weight 1.6 kilos.			
. 1920		per cent	
Apr. 6	Before injection.	81	Animal fasted 2 days previous to an injection of 40 cc. of a 1 per cent solution of nucleic acid = 0.051 gm. of nitrogen.
	2 hrs. after "	70	
	4 " " "	57	
	6 " " "	55	
	8 " " "	53	
" 7	24 " " "	61	
	27 " " "	61	
" 8	48 " " "	65	

An increase in volume, represented by a low hemoglobin would mean a decrease in concentration, and if the non-protein nitrogen did not likewise fall in amount, a real increase of the latter might be indicated. There was some question as to whether the frequent bleeding might not have an effect on the volume; consequently hemoglobin determinations only were made on Rabbit 14 after an injection of nucleic acid. Table VII shows that the volume decrease of about 20 per cent is a result of the injection, and not of the bleeding.

The blood of Rabbit 15 was analyzed for non-protein nitrogen. The results, as given in Table VIII, show an increase of about 5 per cent in the first 3 hours, and in view of the fact that the injection causes a dilution of the blood, the increase is still greater.

Table IX gives a complete picture of the urinary and blood nitrogen after an injection of 0.2 gm. of nucleic acid per kilo in two different rabbits. The urine picture is practically the same as seen in the rabbits previously discussed. The increase in nitrogen on the day following the injection coincides with the accumulation of non-protein nitrogen in the blood within the first 12 hours after injection. In Rabbit 16 the non-protein nitrogen remains practically on the level, but the corresponding decrease in hemoglobin would indicate that there is a marked rise. Rabbit 17 shows a slight increase in non-protein nitrogen, but a decided decrease in hemoglobin, showing a very marked

TABLE VIII.

*Influence of Intravenous Injection of Nucleic Acid on the Non-Protein Nitrogen of the Blood.*

Date.	Non-protein nitrogen.		Remarks.
	Time of drawing blood.	Per 100 cc.	
Rabbit 15. Body weight 1.5 kilos.			
1920		mg.	
Apr. 6	Before injection.	55	Animal fasted 2 days previous to an injection on Apr. 6 of 37 cc. of a 1 per cent solution of nucleic acid = 0.048 gm. of nitrogen.
	3 hrs. after "	61	
	6 " " "	59	
	9 " " "	48	
" 7	24 " " "	56	
	27 " " "	56	
" 8	48 " " "	57	

rise. The marked increase on the last 2 days of the experiment may represent a fasting factor. The animal was very thin and apparently undernourished.

These experiments show that the increase of urinary nitrogen is accompanied by an accumulation of protein catabolic products in the blood.

*The Influence of Repetition of Nucleic Acid Injections upon Nitrogen Excretion in Rabbits.*

Rabbit 11 received the same dose of nucleic acid as was injected a week earlier. The increase in nitrogen output is just as marked or more so, as after the first injection, although the rise comes on



the 2nd day after injection. The phosphates are likewise increased, but the amount of creatine remains practically unchanged, leading one to believe that perhaps immunity is established, as the indications are that the tissue catabolism is not so great. Rabbit 10 shows the result of repeating the dose a month later. The effect is quite similar to that shown for Rabbit 11. The nitrogen output is doubled on the 2nd day after injection, but no marked increase is shown in the creatine.

TABLE X.  
*Repetition of Nucleic Acid Injection.*

Date.	Water intake.	Urine.					Remarks.
		Vol- ume.	Total nitro- gen.	Creati- nine.	Crea- tine.	P <sub>2</sub> O <sub>5</sub>	
Rabbit 11. Body weight 1.8 kilos.							
1920	cc.	cc.	gm.	gm.	gm.	gm.	
Mar. 25	0	60	0.529	0.056	0.006	0.194	Immunity experiment on
" 26	10	35	0.603	0.059	0.041	0.232	Rabbit 7, 1 week later.
" 27	0	52	0.768	0.063	0.012	0.103	Mar. 26, injection
" 28	20	110	1.245	0.058	0.046	0.410	of 22.3 cc. of a 1 per
" 29	0	45	1.578	0.039	0.042	0.308	cent nucleic acid solu- tion = 0.029 gm. of nitrogen.
Rabbit 10. Body weight 2.24 kilos.							
Mar. 17	0	90	1.080	0.057	0.017	0.090	Immunity experiment
" 18	100	100	?	0.066	0.001	0.165	on Rabbit 4, 1 month
" 19	0	125	0.945	0.081	0.009	0.258	later. Mar. 18, injection
" 20	0	135	1.701	0.081	0.024	0.324	of 83.3 cc. of a 1
" 21	100	110	2.300	0.073	0.062	0.187	per cent nucleic acid solution = 0.108 gm. of nitrogen.

#### SUMMARY.

The experiments reported point toward a marked tissue catabolism, chiefly shown in the urinary nitrogen and creatine. There is likewise a rise in the non-protein nitrogen of the blood, which is not so marked. The fact that non-protein nitrogen is being introduced with the nucleic acid injection must be taken into consideration. This may amount to as much as is already

present in the blood as, for example, is the case with Rabbit 15. According to McQuarrie's figures on blood volume, a rabbit of 1.5 kilos would have about 100 cc. (6.5 cc. per 100 gm. of body weight), so that the introduction of 48 mg. of nitrogen into the blood stream would double the amount present, which was 55 mg. per 100 cc. of blood. But with normal kidneys, this should be eliminated quickly. The fact that the non-protein nitrogen does not rise very markedly may be explained by the excretion of the catabolic products about as rapidly as they are formed, so that there would be no great accumulation of these products at any one time. We would suppose this to be the case if the kidneys were working efficiently. The dilution of the blood following nucleic acid injection in the rabbit differs from the results obtained by Ringer and Underhill for the dog. In the dog a marked concentration may be present. It would appear in the rabbit experiments that the relatively large volume of fluid introduced is only slowly compensated for in this animal. In the dog compensation occurs relatively quickly.

#### CONCLUSION.

Injection of nucleic acid into the circulation of the fasting rabbit induces increased tissue catabolism, as indicated by the augmented output of urinary nitrogen and creatine.

In this respect, then, the dog and rabbit respond alike to the intravenous introduction of nucleic acid.

In the rabbit nucleic acid injection produces dilution of the blood, in the dog concentration.

In the rabbit there may be an increased non-protein nitrogen of the blood after nucleic acid injection.





# STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

## XI. THE INFLUENCE OF SOME PROTEIN SPLIT PRODUCTS UPON THE METABOLISM OF FASTING RABBITS.\*

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(Received for publication, August 1, 1921.)

A previous communication<sup>1</sup> relative to the influence of various protein split products upon the metabolism of fasting dogs has shown that in general the higher members of hydrolytic change of protein induce increased catabolism as evidenced by a large output of urinary nitrogen, creatine, and phosphates. This is especially true for such substances as Witte peptone, purified proteoses, and Vaughan's crude soluble poison. In general, however, the dog is peculiarly susceptible to the influence of proteoses and proteose-like substances, and the character of response elicited by the introduction of such substances is sufficiently important in its bearing upon the problems of inflammatory processes to warrant the extension of this type of experiment to a species of animal recognized to be refractory to proteose injection. Such an animal is the rabbit.

### *Methods.*

In general the methods followed were those outlined in a former paper<sup>2</sup> hence repetition is unnecessary.

\* The data are taken from theses presented by Philip Greenberg and Anthony F. Alu in partial fulfillment of the requirements for the degree of Doctor of Medicine, Yale University, 1920.

<sup>1</sup> Ringer, M., and Underhill, F. P., *J. Biol. Chem.*, 1921, xlviii, 503.

<sup>2</sup> Underhill, F. P., and Long, M. L., *J. Biol. Chem.*, 1921, xlviii, 537.

*The Influence of Intravenous Injections of Witte Peptone, and Proteoses upon Nitrogen Excretion.*

An extensive experience with the influence of fasting upon urinary excretion in the rabbit has demonstrated that this con-

TABLE I.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 2.0 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	130	15		Acid.	0.612			0.070	
2	0	27		"	0.662	83	9	0.073	
3	0	35		"	1.068	106	25	0.138	Injection of 0.4 gm. of Witte peptone per kilo (0.158 gm. of nitrogen). Volume 50 cc.
4	0	20		"	0.885	87	8	0.170	
5	110	58	1,030	"	0.969	85	6	0.169	

TABLE II.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 1.8 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	0	62	1,022	Acid.	0.704	815	92	0.070	
2	20	65	1,020	"	0.978	922	66	0.171	
3	25	95	1,021	"	1.038	125	51	0.152	Injection of 0.15 gm. of Witte peptone per kilo (0.05 gm. of nitrogen). Volume 50 cc.
4	25	83	1,024	"	1.716	164	71	0.287	
5	10	80	1,021	"	1.603	149	88	0.265	

dition contributes no complicating factor in the interpretation of results obtained after introduction of various solutions.<sup>2,3</sup> Repetition of such data, therefore, will be omitted.

<sup>3</sup> Also from many unpublished data.

Relative to the toxicity for rabbits of Witte peptone and purified proteoses<sup>4</sup> it may be stated that in no instances was there indication that the animals were even mildly ill. However, several animals died without definite symptoms.

TABLE III.

*The Influence of Witte Peptone on Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1	40	57	1,040	Acid.	0.663	100	21	0.140	
2	90	96	1,021	"	0.675	98	8	0.199	
3	0	295	1,020	"	1.089	114	9	0.215	Injection of 0.4 gm. of Witte peptone per kilo (0.160 gm. of nitrogen). Volume 100 cc.
4	65	64	1,030	"	0.813	98	7	0.236	
5									

TABLE IV.

*The Influence of Deuterocaseose on Urinary Nitrogen Output in a Rabbit of 3.1 Kilos Body Weight.*

Day.	Intake.	Volume.	Specific gravity.	Reaction.	Nitrogen.	Creatinine.	Creatine.	Phosphates.	Remarks.
	cc.	cc.			gm.	mg.	mg.	gm.	
1			Specimen lost.						
2	0	47		Acid.	0.852	131	8	0.263	
3	10	65		"	1.233	181	47	0.314	Injection of 0.15 gm. of deuterocaseose per kilo (0.074 gm. of nitrogen). Volume 50 cc.
4	0	62	1.031	"	0.978	123	34	0.226	
5	100	46		"	0.834	104	28	0.194	

From the view-point of the influence of Witte peptone and purified proteoses upon nitrogenous metabolism the data given in Tables I to IV show quite clearly that there is an increase in

<sup>4</sup> The preparation of this substance has already been described. Cf. Underhill, F. P., and Ringer, M., *J. Pharmacol. and Exp. Therap.*, 1921, in press.

the output of urinary nitrogen. In most cases also there is a definite but smaller augmented excretion of creatine and phosphates. In spite then of the fact that the rabbit and dog show different responses to the acute effects of the introduction into the circulation of Witte peptone and purified proteoses these substances exert with both types of animal the same effect upon nitrogenous metabolism, a fact which would lead one to the view that the influence upon nitrogenous metabolism is not necessarily closely associated with the influence which produces the toxic symptoms.

*The Influence of Vaughan's Crude Soluble Poison and Vaughan's Non-Toxic Body upon Nitrogenous Metabolism.*

In Tables V and VI may be found illustrative data selected from many experiments obtained after injection of Vaughan's crude soluble poison prepared from egg white. An inspection of these tables will make it evident that the intravenous injection of Vaughan's crude soluble poison (neutralized) calls forth a significant increase in the urinary nitrogen output of the fasting rabbit. The augmented nitrogen excretion is accompanied by a corresponding increase in the phosphate elimination. Creatine and creatinine are not markedly influenced.

It will be recalled that in the preparation of Vaughan's crude soluble poison a portion of the protein employed as the source becomes insoluble in the alkaline alcoholic solution and does not possess toxic properties. Such a product obtained from casein when injected into guinea pigs was harmless. On the other hand, the intravenous introduction of this non-toxic substance into the rabbit induces just as great an increase in urinary nitrogen and phosphorus as comparable doses of Vaughan's crude soluble poison (see Tables VII and VIII). In other words the non-toxic material is just as efficacious in accelerating protein catabolism as is the poisonous portion. It must therefore be quite apparent that this influence upon protein catabolism is not specific for Vaughan's crude soluble poison nor is its influence necessarily associated with its toxic properties as evidenced by other symptoms.

TABLE V.

*The Influence of Vaughan's Crude Soluble Poison upon the Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	95	17	Acid.	0.756	0.024	85	11	
2	0	38	"	0.774	0.049	111	11	
3	0	35	"	0.874	0.064	88	17	
4	0	69	"	1.552	0.124	158	48	
5	0	50	"	1.050	0.120	160	50	Injection of 105 mg. of Preparation I ( = 0.10 gm. of nitrogen) dissolved in 100 cc. of 0.9 per cent NaCl = 50 mg. per kilo of body weight. Time, 10 minutes.

TABLE VI.

*The Influence of Vaughan's Crude Soluble Poison upon the Urinary Nitrogen Output in a Rabbit of 1.8 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	42	52	Acid.	0.732	0.215	82	4	
2	30	68	"	0.762	0.179	65	10	
3	5	114	"	1.018	0.250	62	35	
4	0	28	"	0.693	0.163	65	69	
5	0	50	"	0.640	0.160	63	58	Injection of 135 mg. of Preparation I ( = 0.14 gm. of nitrogen) dissolved in 0.9 per cent NaCl = 75 mg. per kilo of body weight. Time, 10 minutes.

TABLE VII.

*The Influence of Vaughan's Non-Toxic Protein upon the Urinary Nitrogen Output in a Rabbit of 2.1 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	0	54	Acid.	0.600	0.100	90	8	Injection of 210 mg. of non-toxic protein ( = 0.23 gm. of nitrogen) dissolved in 0.9 per cent NaCl = 100 mg. per kilo of body weight. Time, 10 minutes.
2	0	42	"	0.780	0.184	87	15	
3	0	122	"	1.200	0.600	99	53	
4	0	78	"	1.398	0.425	93	74	
5	0	80	"	2.432	0.425	88	92	

TABLE VIII.

*The Influence of Vaughan's Non-Toxic Protein upon the Urinary Nitrogen Output in a Rabbit of 2.3 Kilos Body Weight.*

Day of fasting.	Water intake.	Urine.						Remarks.
		Volume.	Reaction to litmus.	Total nitrogen.	Phosphates $P_2O_5$ .	Creatinine.	Creatine.	
	cc.	cc.		gm.	gm.	mg.	mg.	
1	40	62	Acid.	0.810	0.210	69	21	Injection of 115 mg. of non-toxic protein ( = 0.12 gm. of nitrogen) dissolved in 50 cc. of 0.9 per cent NaCl = 50 mg. per kilo of body weight. Time, 10 minutes.
2	0	45	"	0.880	0.165	64	33	
3	0	47	"	1.014	0.265	88	42	
4	0	45	"	0.942	0.238	72	55	
5	0	46	"	1.200	0.240	70	70	

## CONCLUSIONS.

In spite of the fact that the rabbit is refractory to the acute effects of Witte peptone and proteoses, the intravenous injection of these substances into the fasting animal induces an accelerating influence upon protein catabolism similar to the response obtained in dogs.

A similar influence is exerted by the intravenous injection of Vaughan's crude soluble poison and Vaughan's non-toxic body.

These facts lead to the view that the action exerted upon nitrogenous metabolism is not necessarily related to toxic properties possessed by some of these substances.

The influence seen upon nitrogenous metabolism is therefore not specific for a given protein derivative but is probably an indication of the detrimental action incident to the introduction into the circulation of a foreign protein.





# THE INFLUENCE OF THYROPARATHYROIDECTOMY UPON BLOOD SUGAR CONTENT AND ALKALI RESERVE.

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(Received for publication, August 1, 1921.)

In a recent communication Hastings and Murray<sup>1</sup> report their failure to corroborate the work of Underhill and Blatherwick<sup>2,3</sup> who showed that blood sugar content is low after thyroparathyroidectomy. They state that "their (Underhill and Blatherwick) determinations made with the method of Forschbach and Severin showed extraordinary variations and were not accompanied by sufficient data to allow for much comment." In reply it may be stated that the only data essential for the understanding of the problem were the changes in blood sugar content and their correlation to the occurrence of tetany and these were given in full. No mention is made of the experiments of Underhill and Blatherwick<sup>2</sup> wherein blood sugar estimations were made by the method of Vosburgh and Richards<sup>4</sup> which involves the actual weighing of cuprous oxide. It is quite fair to admit that results obtained by the Forschbach and Severin method may be subject to criticism because of the small quantity of blood employed in this colorimetric procedure. No such criticism can, however, be applied to the method of Vosburgh and Richards especially since quantities of blood varying from 20 to 60 gm. were employed for sugar estimations.

Hastings and Murray employed the MacLean method of sugar estimation and assert that "in our series, at least, there was no marked disturbance in sugar metabolism for the first few days

<sup>1</sup> Hastings, A. B., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlv, 233.

<sup>2</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.

<sup>3</sup> Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xix, 119.

<sup>4</sup> Vosburgh, C. H., and Richards, A. N., *Am. J. Physiol.*, 1903, ix, 35.

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after operation." Their protocols agree with their conclusions. The divergence of results appears to demand a reinvestigation of the problem and the results of four experiments are recorded.

### *Methods.*

Well nourished adult dogs were maintained in a fasting condition throughout the investigation. The operation was performed under morphine-atropine-ether anesthesia, the entire thyroid-parathyroid apparatus being removed. Blood was drawn from an ear vein. Blood sugar estimations were made according to the method of Folin and Wu.<sup>5</sup> Carbon dioxide capacity of the plasma was measured by the procedure of Van Slyke. As a rule determinations on the blood were made about 9.30 a.m. and 5 p.m. daily.

### *Does Hypoglycemia Occur After Removal of the Thyroids and Parathyroids in Dogs?*

It is quite evident from the data in the table that after complete removal of the thyroids and parathyroids there is a variable but distinct fall in the blood sugar content. This usually occurs after the onset of tetany but may be present before signs of tetany are apparent.

The reason for the difference in the results of Hastings and Murray and our own is not evident unless indeed it is related to the question of nutrition. In all our work with the exception of a single dog the animals were maintained in a fasting state whereas in the investigation of Hastings and Murray the context of their paper would lead one to the conclusion that food was given (*cf.* p. 240). That such an explanation is probably inadequate may be inferred from the fact that in Experiment 3 of our first paper<sup>2</sup> the animal ate food for a period of 12 days after operation, then went into tetany and revealed a condition of hypoglycemia.

With the idea in mind that changes in blood concentration might possibly play a rôle in the topic under discussion hemoglobin and total solid values in the blood were followed. These observations indicate perceptible alterations in concentration from time to time but since these values fluctuate in either direc-

<sup>5</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

tion without apparent relationship to tetany or blood sugar content they are without definite significance and hence are omitted here.

The work has now been repeated three times employing three different methods for sugar estimation, and whatever may be the reason for the divergent results we see no occasion for modifying our former conclusion; namely, that removal of the thyroids and parathyroids in dogs induces a condition of lowered blood sugar content.

*The Relation of Alkali Reserve to Removal of the Thyroids and Parathyroids.*

In another section of their communication Hastings and Murray state that after removal of the thyroids and parathyroids there is not the slightest evidence of an alkalosis as indicated by a study of the carbon dioxide capacity of the plasma. This conclusion is directly opposed to the work of Wilson, Stearns, and Thurlow<sup>6</sup> published in 1915. These investigators found by determination of Barcroft's dissociation constant of oxyhemoglobin in venous blood brought into equilibrium with a constant tension of carbon dioxide and measurements of the carbon dioxide tension in the alveolar air evidence of an increasing alkali reserve up to the onset of tetany. McCann<sup>7</sup> later published experiments substantiating this. In view of the importance of the theoretical considerations involved it seemed very desirable to investigate further the problem since the results of Hastings and Murray fail to corroborate the views of Wilson and his colleagues. Advantage has therefore been taken of the opportunity afforded by the animals prepared for the previous work on blood sugar.

From the data presented in the table it must be concluded that up to the onset of tetany little or no change occurs in the alkali reserve of the blood after removal of the thyroids and parathyroids. It is true that with Dog 2 the carbon dioxide capacity is greater the day after the operation than previously. The change is slight, however, and probably little significance should be attached to it, since even greater changes may occur in normal

<sup>6</sup> Wilson, D. W., Stearns, T., and Thurlow, M. De G., *J. Biol. Chem.*, 1915, xxiii, 89.

<sup>7</sup> McCann, W. S., *J. Biol. Chem.*, 1918, xxxv, 553.

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dogs in a fasting condition. This may be seen from data taken from another investigation in which dogs were subjected to a preliminary period of fasting. The following figures are for simple fasting, no other procedure having been followed with the animals concerned.

Days of fasting.....	1	2	3
CO <sub>2</sub> capacity (Dog 1).....	54	68	65
CO <sub>2</sub> " ( " 2).....		51	60

After the onset of tetany in general there may be a decided tendency toward a diminished alkali reserve.

### CONCLUSIONS.

In spite of contrary findings by Hastings and Murray, repetition of previous experiments leads to the reiteration of a former conclusion; namely, that thyroparathyroidectomy results in a lowered blood sugar content.

After this operation there seems to be little or no change in the carbon dioxide capacity of the blood up to the onset of tetany. After this period there may be a decided tendency toward a diminished alkali reserve.

### *The Influence of Thyroparathyroidectomy upon Blood Sugar Content and Alkali Reserve.*

Date.	Blood sugar per 100 cc.	Plasma CO <sub>2</sub> capacity.	Remarks.
<b>Dog 1.</b>			
1921	mg.		
Apr. 20	109	a.m.	70.8 a.m. Normal.
	111	p.m.	68.9 p.m.
" 21	111	a.m.	Normal.
" 25	93	"	67.3 " Removed thyroids and parathyroids 3 p.m. Apr. 25.
" 26	149	"	Appears normal.
	131	p.m.	58.2
" 27	112	"	55.3 Slight tetany.
" 28	91	a.m.	63.6
	78	(4.30 p.m.)	58.2 Marked tetany. Died in
	58	(5.30 " )	44.7 convulsions at 6.30 p.m.

*The Influence of Thyroparathyroidectomy—Concluded.*

Date.	Blood sugar per 100 cc.	Plasma CO <sub>2</sub> capacity.	Remarks.
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**Dog 2.**

1921	mg.		vol. per cent	
May 25	99	p.m.	53.6	Normal.
" 26	98	a.m.	52.9	" Removed thyroids and parathyroids at 3 p.m.
" 27.	117	"	60.2	Appears normal.
	119	p.m.	58.3	
" 28	45	a.m.	30.9	Severe tetany.
	37	p.m.	44.7	" " Died in convulsions.

**Dog 3.**

June 6	103	p.m.	60.3	Normal.
" 7	96	a.m.	54.3	" Removed thyroids and parathyroids 3 p.m.
" 8	108	"	57.7	Runs, normal.
	101	p.m.	46.4	
" 9	111	a.m.	52.6	
	96	p.m.	48.8	Slight tetany.
" 10	100	(10 a.m.)	54.0	
	101	(11 " )	46.8	Severe tetany.
	67	(10 p.m.)	58.8	
" 11	74	a.m.	49.7	Tetany absent.
	73	p.m.	47.7	Dog comatose.
" 12	85	a.m.	47.7	" "
	67	p.m.	51.4	
" 13	107	a.m.	51.4	Dog comatose.
	93	p.m.	48.3	

**Dog 4.**

June 27	111	a.m.	51.7	Normal. Removal of thyroids and parathyroids at 3 p.m.
" 28	106	"	49.9	Seems normal.
	100	p.m.	49.9	
" 29	83	a.m.	51.7	Seems normal.
	95	p.m.	49.9	
" 30	81	"	54.1	Slight tetany.
July 1	104	(9.30 a.m.)	42.5	Marked tetany.
	75	(11.30 " )	34.9	" "
	80	(3.00 p.m.)	32.9	Tetany subsiding.
" 2	94	(8.30 a.m.)	46.8	No tetany.
	96	(Noon.)	46.8	



# THE INFLUENCE OF FOOD INGESTION UPON ENDOGENOUS PURINE METABOLISM. I.

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(Received for publication, August 24, 1921.)

Abundant evidence has been accumulated in recent years to indicate that the original idea of Burian and Schur (1) and Sívén (2), to the effect that the excretion of endogenous purines is constant from day to day in the same individual, is not correct. More than 15 years ago Folin (3) found that the change from a diet of milk and eggs to one of starch and cream might be accompanied by a fall in uric acid elimination of practically 50 per cent, although both diets are purine-free. He stated that the uric acid excretion is reduced whenever the total nitrogen elimination is much diminished, but that the reduction is irregular, and variable for different individuals.

Since the work of Folin, numerous investigators, notably Leathes (4), Mendel and Brown (5), Smetánka (6), Taylor and Rose (7), Mendel and Stehle (8), Lewis and Doisy (9), and Höst (10), have corroborated his findings that protein ingestion exerts a marked influence upon urinary uric acid. Smetánka (6), Mendel and Stehle (8), and Umeda (11) also observed an increase in uric acid elimination, as compared with the fasting output, following the ingestion of carbohydrates. Apparently fats produce the least effect upon purine metabolism of either of the three food-stuffs.

The calorific value of the diet is likewise important in determining the uric acid excretion. According to Graham and Poulton (12), diets of protein and fat of insufficient calorie value, cause a fall of 30 to 50 per cent in the output of endogenous uric acid. If most of the fat is replaced by carbohydrate no fall is observed. More recently Höst (10) has affirmed that every increase or



decrease in the calorific value of the food beyond a certain minimum, is accompanied by a like change in uric acid excretion. This effect occurs no matter which foodstuff is responsible for the variation in energy value of the diet, but is greatest with changes due to protein.

No unanimity of opinion exists, however, as to the cause of the increases and decreases in endogenous uric acid elimination induced by diet. Various factors have been held responsible, and at least six possible explanations have been suggested or advocated in an effort to explain the experimental observations. It is our purpose in this paper to briefly discuss these theories. In the succeeding communication data will be presented which we believe throw additional light on the problem.

Alterations in the excretion of endogenous uric acid resulting from variations in the kind and amount of food have been attributed to the following factors: (1) Nuclear disintegration in the glands of the alimentary canal occasioned by the work of digestion (Mareš (13), Smetánka (6), Lambling and Dubois (14), Mendel and Stehle (8), Höst (10)). (2) Nuclear disintegration associated with the work of digestion and food storage (Smetánka, 6). (3) Synthesis of purines from carbohydrates (Graham and Poulton (12), Umeda (11)). (4) Synthesis of purines from arginine and histidine (Ackroyd and Hopkins (15), Harding and Young (16)). (5) Stimulation of the process of elimination (suggested by Lewis, Dunn, and Doisy (17), but regarded by them as untenable). (6) General stimulation of cellular metabolism by amino-acids or their catabolic derivatives (Lewis, Dunn, and Doisy, 17).

The first of these theories was suggested by Mareš (13). This investigator attributed the increase in output of uric acid following the consumption of purine-free food to nuclear disintegration, chiefly in the alimentary glands, incidental to the physiological work of secretion and digestion. Uric acid thus represents, according to Mareš, the wear and tear of these glandular tissues. When the alimentary glands are resting, the output of uric acid is low; when they are actively synthesizing and secreting digestive fluids, wear and tear is increased, and the production of uric acid is accelerated. As further evidence for this mechanism, Mareš points to the fact that pilocarpine, which is known to increase secretory activity, likewise augments the excretion of uric acid.

Smetánka (6), Lambing and Dubois (14), and Höst (10) also regard digestive work as an important factor in the variations in output of endogenous uric acid. Mendel and Stehle (8), on the other hand, state that their experiments "offer no obstacle to the assumption that *a portion, at least, of the endogenous uric acid may originate from the activity of the alimentary secretory apparatus.*" The latter investigators were able to confirm the findings of Mareš in regard to the action of pilocarpine, and to make the additional observation that atropine, which diminishes secretory activity, causes a fall in uric acid elimination.

The Mareš theory seems inadequate to us for several reasons. It does not appear probable that disintegration of the secretory cells during the process of digestion would be sufficiently extensive to account for the increase in uric acid elimination, which in some case is relatively large (*cf.* Taylor and Rose (7)). It is true that the suggestion of Mendel and Stehle (8) and Höst (10), to the effect that perhaps only a part of the uric acid has its origin in the alimentary cells, obviates this difficulty. Such a suggestion, however, does not afford an explanation of the effect of amino-acids, which require no digestion, upon uric acid elimination. Lewis, Dunn, and Doisy (17) have shown that glycocoll, alanine, and other amino-acids increase the hourly output of uric acid during fasting as much as do proteins. In our own experiments described in the succeeding paper, the protocols show that even though the subjects lived upon weighed diets, necessitating the daily expenditure of the same amount of physiological labor in the process of digestion, the uric acid output for the individual days of the periods was quite variable.

Smetánka (6), who in the main adheres to the Mareš theory, was forced to modify it in view of results obtained by him in experiments somewhat similar in nature to those of Lewis, Dunn, and Doisy. Having observed that the ingestion of honey, a food which like amino-acids requires practically no digestion, causes a marked increase in the output of uric acid, this investigator suggested that in addition to digestive work, the activity involved in glycogenesis may be responsible for a part of the endogenous uric acid. As far as the writer is aware, this is the only suggestion in the literature which specifically attributes uric acid formation in part to the process of food storage. While the theory is

interesting, and perhaps comes nearer explaining the experimental facts than does the original Mareš conception, still it is open to the same criticism as regards the action of amino-acids. If the increased output of uric acid following the ingestion of honey is due to the increased glycogenesis, certainly some other factor must be responsible for the action of glycocoll, alanine, and other compounds which cannot form appreciable quantities of this polysaccharide.

In connection with Smetánka's observation concerning the effect of honey, the investigations of Graham and Poulton (12) and of Umeda (11) are of interest. These authors believe that part of the endogenous uric acid may arise through synthesis from carbohydrates. They observed that carbohydrate-rich fat-poor diets cause a greater excretion of uric acid than do fat-rich carbohydrate-poor diets, even though the protein content and calorific value of the food are maintained constant. Umeda suggests that uric acid may arise from a condensation of urea with an intermediary product of carbohydrate metabolism, perhaps lactic acid. As evidence for a synthesis of purines from carbohydrates, Graham and Poulton point to the observation of Knoop and Windaus (18) that when glucose is exposed *in vitro* to the action of sunlight and the strongly dissociated compound,  $\text{Zn}(\text{OH})_2 \cdot 4\text{NH}_3$ , methyl glyoxal and 5-methyl-imidazole are formed. As interesting as these suggestions are, there exists at the present time no experimental evidence *in vivo* which justifies the belief that carbohydrates are transformed into purines in the animal organism. The observations of Ackroyd and Hopkins (15) which are discussed below indicate that in the rat, at least, purine synthesis from carbohydrate, if it occurs at all, is not quantitatively sufficient to meet the demands of the growing organism for these nuclear constituents.

One of the most important studies of endogenous purine metabolism of recent years is the paper of Ackroyd and Hopkins (15) alluded to above. These authors found that when young rats were supplied diets deprived of arginine and histidine, but adequate in every other respect to meet the demands of growth, growth ceased and the elimination of allantoin decreased 40 to 50 per cent. When either arginine or histidine was present in the diet, there was no loss of weight, and in some cases growth oc-

curred. The decrease in allantoin excretion was likewise much less than when both diamino-acids were absent from the food. No fall in allantoin elimination occurred when tryptophane was removed from the ration, or as a result of the absence of a vitamin supply, though nutritional failure in these cases was even greater than when arginine and histidine were withheld. Despite the difficulties which are obviously associated with metabolic studies involving quantitative urine analyses in small animals, the care with which the experiments of Ackroyd and Hopkins are controlled, and the uniformity of their results, justify, in our opinion, their conclusions that arginine and histidine are the most readily available raw materials for purine anabolism in the body. Apparently either of these amino-acids may serve as the substrate for purine formation.

A similar conclusion as to the origin of purines in the diamino-acids was recently arrived at by Harding and Young (16). According to these investigators, the feeding of placenta, which has a high content of arginine, causes a much greater increase in the output of uric acid and allantoin in young dogs than does the ingestion of an equal quantity of muscle protein. Inasmuch as the diets of their animals were not purine-free, the data of Harding and Young are not as convincing as those of Ackroyd and Hopkins.

On the contrary, Abderhalden and Einbeck (19), Abderhalden, Einbeck, and Schmid (20), and Lewis and Doisy (9) have been unable to show any relationship between the arginine and histidine content of the diet and the uric acid or allantoin output in the urine. Lewis and Doisy compared the effects of diets high and low in arginine and histidine upon the uric acid output in man. Abderhalden and Einbeck studied the effects of adding histidine to the diet upon the allantoin excretion in the dog. In the later experiment of Abderhalden and his coworkers (20), histidine hydrochloride was given in 10 gm. doses to a fasting animal. Neither of the experiments yielded any evidence for an origin of purines in the diamino-acids. We believe the procedures made use of by these investigators were not suitable for studying the relation of amino-acids to purine syntheses. In the experiments of Lewis and Doisy (9), it is quite possible that the "low" histidine-arginine diets contained adequate amounts of purine pre-

cursors to support the normal anabolism. Calculation from the authors' data shows that the "low" diets contained 3.5 to 4.0 gm. of arginine and histidine in each day's ration. If such amounts are adequate (we have no information as to the quantities of diamino-acids required by adult men), one would hardly expect that a more abundant supply would result in an exaggerated purine synthesis, and an increased uric acid elimination. We shall return to this proposition later.

Concerning the experiments of Abderhalden, the question is properly raised by Ackroyd and Hopkins (15) as to whether an abnormal condition like fasting affords the best opportunity for investigating the fate of an amino-acid. They believe<sup>1</sup> that the

" . . . synthesis of such essential tissue constituents as the purines continues during starvation, at the expense—as we are entitled to believe—of protein materials liberated by autolysis of the less essential organs. When however an excess of a single amino-acid enters the circulation of a starving animal in a single isolated dose it may well almost completely escape such special utilization. It appears suddenly in excess of current needs, and, because the processes of deamination and direct oxidation are always in action, it will almost certainly survive for but a short period as available material for synthesis."

In contrast to the methods of Abderhalden and Lewis and their coworkers, Ackroyd and Hopkins compared the effects of diets *free* from arginine and histidine, with diets containing *adequate amounts* of the diamino-acids. The importance of this procedure is emphasized by them as follows:<sup>2</sup>

"When an animal is in a state of full nutrition it does not follow that such a process as the synthesis of the purine ring would necessarily be much accelerated or increased by mere increase in the supply of its raw material."

And again,<sup>2</sup>

"An individual amino-acid fed in excess of the immediate current needs of the tissues, as when it is added to an already efficient dietary, will almost certainly be rapidly broken down on more direct lines, even if it be a normal precursor of the purine (or other) synthesis in the body."

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<sup>1</sup> Ackroyd and Hopkins (15), pp. 552 and 553.

<sup>2</sup> Ackroyd and Hopkins (15), p. 552.

As important as the investigations of Ackroyd and Hopkins appear to us, we do not believe that they, or other studies of their kind, have an immediate bearing upon the problem of the variations in endogenous uric acid elimination incident to alterations in the kind and amount of purine-free food, *when the amino-acids in question are included in the diet*. Even if it be admitted, as we are prepared to do, that tissue purines have their ultimate origin in arginine and histidine, this fact, in our opinion, does not warrant making the assumption that the extent of purine synthesis is proportional to the arginine-histidine supply. On the contrary, it seems reasonable to suppose that the anabolism of any tissue component is limited quantitatively to the needs of the organism for that particular ingredient. As soon as a diet contains sufficient precursors of any given anabolic product, synthesis of that product at the optimum rate probably occurs. It seems unlikely that the optimum would be exceeded however great a redundancy of the precursors in question were provided. We believe that this view is entirely in accord with the statements of Ackroyd and Hopkins quoted above, and is completely justified in the case of purine anabolism by the experiments of Abderhalden and Lewis and Doisy. If we are correct, one should no more expect to exaggerate purine anabolism by feeding excessive quantities of purine precursors, than he should anticipate being able to increase the mass of brain substance by feeding unusual amounts of the components of nervous tissue. With the exception of the purely storage forms of foods (glycogen, fats, and to a less extent, amino-acids), the components of the tissues of each species are normally synthesized and retained in remarkably uniform proportions. If conditions were otherwise, tissue composition would be largely determined by the accident of diet rather than by the expression of the inherent, hereditary tendencies and impulses of the organism. It is, therefore, rather surprising to us that Harding and Young (16) were able to note differences in purine excretion in pups on diets of placenta (high in diamino-acids) as contrasted with diets of muscle (low in diamino-acids), unless the differences were in part due to exogenous purines. On the other hand, the fact that in their experiments growing animals were used, in which the anabolic reactions are known to predominate, may have been responsible for their unique data.

In accordance with these concepts, *instead of there being conflict between the data of Ackroyd and Hopkins on the one hand, and Lewis and Doisy on the other, we regard them as entirely in accord and mutually supplementary.* In the experiments of the former, the decrease in allantoin excretion following the removal of arginine and histidine from the diet is the significant point, rather than the increase, which probably represented the normal purine metabolism, when the diamino-acids were supplied. After removal of arginine and histidine from the diet growth ceased because purine (and perhaps other) anabolism was no longer possible. Because of the deficient anabolism, greater physiological economy was exercised in catabolism, and the catabolic end-product of purines in the rat (allantoin) decreased in amount. In the experiments of Lewis and Doisy, a high arginine-histidine diet *failed to induce a greater elimination* of the catabolic end-product of purines in man (uric acid) than did a low arginine-histidine diet, because the latter was adequate to permit the optimum anabolism of purines. The superfluous molecules of arginine and histidine were doubtless oxidized without passing through the purine stage. In other words, the work of Ackroyd and Hopkins, to our mind, renders it very probable that the ultimate sources of tissue purines are arginine and histidine; the investigation of Lewis and Doisy indicates that purine anabolism in the adult is limited in extent to the physiological needs of the organism for purines. Neither investigation, however, permits any conclusions to be drawn as to the cause of variations in purine elimination with diets containing adequate amounts of diamino-acids. The latter problem is more likely one of purine catabolism or excretion rather than of anabolism.

In the course of the exceedingly interesting investigation of Lewis, Dunn, and Doisy (17) on the influence of diet upon the hourly elimination of uric acid, the possibility occurred to the authors that the increased uric acid excretion following the ingestion of a single dose of a protein or of an amino-acid might be due to a *stimulation of the processes of excretion* under the influence of the food, rather than to increased uric acid formation. They reasoned that if a single dose of an amino-acid produced its effect by bringing about the mobilization and elimination of reserve or stored purines or their precursors, the administration of a

second dose, after the effect of the first had reached its maximum, should be without further influence. Accordingly, an experiment was made in which successive doses of glycocholate were administered on the same experimental day. The figures show that entirely comparable increases in the hourly output of uric acid occurred after each dose. According to the authors the data<sup>3</sup> "clearly demonstrate that the effects of amino-acids on uric acid excretion are not the result of stimulation of excretory processes leading to a removal of preformed uric acid from the body." While it might be questioned whether Lewis, Dunn, and Doisy were justified in assuming that the first dose of glycocholate entirely removed *all* excess or reserve purines from the system, and whether the single experiment reported by them is sufficient to warrant their conclusion in this regard, data of another sort in the literature, when considered in connection with their work, increase the probability of their contention being well founded. Frequent estimations of uric acid in the blood of normal subjects upon widely different purine-free diets led Höst (10) to the conclusion that diet (in the absence of purines) is without influence upon the concentration of uric acid in the blood. Despite the fact that urinary uric acid varies greatly in a given individual as a result of changes in the composition of the food, the proportion in the blood remains constant within the experimental error of the method. Inasmuch as uricolysis is not believed to occur in the human subject, and since the concentration of uric acid in normal blood is invariable under the influence of purine-free food, Höst is of the opinion<sup>4</sup> that "the endogenous uric acid output becomes a direct expression for the uric acid formation." It must be admitted that rather large quantities of uric acid would have to be retained in the blood in order to alter appreciably the proportion present, or that reserve purines in the sense of Lewis, Dunn, and Doisy might be stored in the tissues, and hence not be manifested by blood analyses at all. Nevertheless, such evidence as we have, whether obtained from a study of the urine (Lewis, Dunn, and Doisy), or by means of blood analyses (Höst), indicates that the cause of the alterations in output of endogenous

<sup>3</sup> Lewis, Dunn, and Doisy (17), p. 17.

<sup>4</sup> Höst (10), p. 30.



uric acid following food consumption is not to be sought in an exaggerated excretion.

Having excluded to their own satisfaction the possibility of a stimulation in excretion being the causative factor in the increased output of uric acid following protein ingestion, Lewis, Dunn, and Doisy (17) suggest that the effect may be due to a general stimulation of all cellular metabolism by amino-acids. Each of the four amino-acids, glycocoll, alanine, glutaminic acid, and aspartic acid, as well as the closely related asparagine, caused an appreciable increase in the hourly fasting output of uric acid. The stimulation caused by the dicarboxylic amino-acids was more marked than that produced by glycocoll and alanine. On the other hand, sarcosine, a substituted amino-acid not readily catabolized by the body, and ammonium chloride and urea, were without influence. The authors emphasize the similarity of the effects produced by protein and amino-acid ingestion upon uric acid formation and heat production (specific dynamic action), and point out that the same chemical factors may be responsible for both.

We believe that there are no experiments in the literature the results of which invalidate the assumption that general stimulation of cellular catabolism, involving both the nuclear purines and the hypoxanthine of muscle tissue, by amino-acids is at least one of the important factors in endogenous purine metabolism. Particularly do the data of Smetánka (6), Taylor and Rose (7), Mendel and Stehle (8), and Höst (10) lend support to this hypothesis. In the succeeding paper we shall present the results of observations which we believe afford additional reasons for accepting this view.

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## THE INFLUENCE OF FOOD INGESTION UPON ENDOGENOUS PURINE METABOLISM, II.

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The investigations reported in this communication have been in progress recurrently for the past 5 years. At first we made numerous studies of the hourly elimination of uric acid in fasting, and following the ingestion of single meals of purine-free foods. The results obtained were, for the most part, similar to those of other investigators who have studied uric acid excretion during short periods, notably, Mendel and Stehle (1), Neuwirth (2), and Lewis, Dunn, and Doisy (3). Invariably, the consumption of food led to an increase in uric acid excretion. But despite every care in the conduct of the experiments, the increases were sometimes so slight, and the fluctuations without the influence of food were relatively so large, that we regarded the interpretation of the data as liable to serious error. We finally abandoned hourly studies and adopted 24 hour periods, which yielded more consistent results.

### *Methods.*

The plan of the investigation was similar to that pursued in a study of endogenous creatine-creatinine metabolism, the results of which were reported in a former communication (4). In the earlier experiments, the uric acid and total nitrogen excretion were determined in subjects ingesting diets alternately low and high in protein. Later, two additional series of studies were undertaken. In one series, the nitrogen intake was maintained constant throughout, while the calorific value of the diet was altered. In the other, a low protein-low calorific diet was alternated with a high protein-high calorific diet.

The subjects were four healthy young men, students in the School of Medicine of this University, who were engaged in the usual routine of student life, with practically the same amount of physical activity each day throughout the experiments. By making use of several individuals, we have excluded the possibility of our findings being accidental, or due to metabolic peculiarities of a single subject. Each student had been subsisting upon a purine-free diet for several days preceding the experimental régime. The diets were prepared, weighed, and ingested in the laboratory, and the meals were served at regular hours three times daily. Urines were preserved with toluene, and were usually

TABLE I.  
*Composition of Diets.*

No. of diet.	Articles of diet.							Food values.*	
	Bread.	Honey	Butter.	Eggs.	Milk.	Cheese	Apples	N	Calories.
	gm.	gm.	gm.	gm.	cc.	gm.	gm.	gm.	
1	400	150	150					6.17	2,770
2	150			655	1,500	50		27.93	2,741
7	400		20	200		50		12.91	1,780
8	400	75	150	100	790		300	12.86	3,433
9	300	150	60				300	4.75	1,978
10	300		90	450	1,500	90	300	27.45	3,907

\* The nitrogen and calorific values here tabulated were calculated from data given by Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Office Exp. Stations, Bull. 28* (revised), 1906.

analyzed immediately after the end of the 24 hour periods. Total nitrogen was estimated by the Kjeldahl-Gunning method, and uric acid by the procedure of Benedict and Hitchcock (5). Table I indicates the composition, nitrogen content, and calorific value of each diet, all of which were of course practically purine-free.<sup>1</sup>

<sup>1</sup> White bread, which contains larger traces of purines than any other article of food included in the diets, was kept constant in quantity throughout each experiment with the exception of those recorded in Tables II and III. In these two, more bread was ingested during the low than during the high diet periods. Accordingly, the effect of traces of exogenous purines, if appreciable at all, would be more pronounced during the periods of low diet, and therefore cannot impair the validity of our conclusions.

## EXPERIMENTAL.

The results of the numerous experiments which we have conducted are entirely in accord with each other, and therefore need not all be detailed in this communication. The protocols give

TABLE II.

*The Influence of High and Low Protein Diets on Uric Acid Excretion, when the Calorific Intake is Constant.*

Experiment 8. Subject F. W. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1916	kg.	cc.		gm.	gm.	
Dec. 7	72.5	1,310	Acid.	6.97	0.40	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.*
" 8		1,550	"	7.38	0.47	
" 9		1,510	"	7.12	0.41	
" 10		2,450	"	7.96	0.44	
" 11		1,180	"	6.78	0.43	
Average.....				7.24	0.43	
Dec. 12	71.6	1,675	Acid.	13.00	0.51	High protein diet, No. 2. 27.93 gm. N and 2,741 calories daily.
" 13		2,390	"	16.49	0.49	
" 14		2,780	"	20.49	0.46	
" 15		2,440	"	22.00	0.51	
" 16		2,690	"	22.78	0.48	
Average.....				18.95	0.49	
Dec. 17	71.6	2,765	Acid.	15.06	0.40	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.
" 18		1,340	"	9.91	0.39	
" 19		1,400	"	8.74	0.39	
Average.....				11.24	0.39	
1917						
Jan. 18	71.6	1,530	Acid.	9.03	0.29	Starvation level. <sup>†</sup>

\* Began eating this diet on Dec. 5.

† Subject ate a purine-free diet for 10 days, followed by a fast of 40 hours. Analyses represent urine of last 24 hours of the fast.

the data of six experiments, two of each of the three series indicated above. In Tables II and III are shown the effects of alternately feeding low protein and high protein diets when the calorific intake is maintained constant. Although there is some

irregularity in the uric acid elimination, as is almost invariably the case with this urine constituent, nevertheless, there is a slight but unmistakable increase in output during the high protein periods. The return to the low protein diet, following the days

TABLE III.

*The Influence of High and Low Protein Diets on Uric Acid Excretion, when the Calorific Intake is Constant.*

Experiment 9. Subject H. L. B.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1916	kg.	cc.		gm.	gm.	
Dec. 7	68.6	400	Acid.	6.73	0.45	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.*
" 8		460	"	6.96	0.51	
" 9		450	"	6.62	0.54	
" 10		730	"	7.26	0.56	
" 11		520	"	6.42	0.54	
Average.....				6.80	0.52	
Dec. 12	68.8	980	Acid.	12.45	0.66	High protein diet, No. 2. 27.93 gm. N and 2,741 calories daily.
" 13		960	"	16.33	0.56	
" 14		980	"	19.02	0.52	
" 15		1,160	"	21.74	0.54	
" 16		1,100	"	22.18	0.55	
Average.....				18.35	0.57	
Dec. 17	69.1	635	Acid.	12.89	0.47	Low protein diet, No. 1. 6.17 gm. N and 2,770 calories daily.
" 18		460	"	8.86	0.48	
" 19		450	"	8.21	0.46	
Average.....				9.99	0.47	
1917						
Jan. 18	69.5	710	Acid.	9.36	0.43	Starvation level. <sup>†</sup>

\* Began eating this diet on Dec. 5.

† Subject ate a purine-free diet for 10 days, followed by a fast of 40 hours. Analyses represent urine of last 24 hours of the fast.

of high protein ingestion, is in each case accompanied by a fall in uric acid excretion. At the bottom of each table is recorded the subject's fasting output of uric acid. As indicated in the protocols, purine-free diets were ingested for 10 days preceding

the 40 hour fasts. The urines for the last 24 hours of the abstinence periods were used for the analyses. In Experiment 8, the starvation uric acid excretion dropped to 0.29 gm., and in Experiment 9, to 0.43 gm. per day.

TABLE IV.

*The Influence on Uric Acid Excretion of Diets High and Low in Calories, when the Protein Intake is Constant.*

Experiment 15. Subject J. S. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1917	kg.	cc.		gm.	gm.	
Dec. 7	62.2	685	Acid.	11.63	0.41	Low calorific diet, No. 7. 12.91 gm. N and 1,780 calories daily.*
" 8		865	"	13.33	0.47	
" 9		1,000	"	12.35	0.49	
" 10		560	"	11.66	0.47	
" 11		615	"	12.56	0.47	
" 12		620	"	12.20	0.54	
Average.....				12.29	0.48	
Dec. 13	61.4	625	Acid.	11.15	0.50	High calorific diet, No. 8. 12.86 gm. N and 3,433 calories daily.
" 14		825	"	11.21	0.58	
" 15		1,490	"	11.17	0.59	
" 16		800	"	9.16	0.55	
Average.....				10.67	0.56	
Dec. 17	61.5	650	Acid.	10.84	0.55	Low calorific diet, No. 7. plus 300 gm. apples.† 13.10 gm. N and 1,971 calories daily.
" 18		890	"	12.30	0.56	
" 19		995	"	12.35	0.57	
" 20		1,175	"	11.93	0.57	
Average.....				11.86	0.56	

\* Began eating this diet on Dec. 3.

† The apples were added because of their laxative properties.

Tables IV and V show the effects of alternately feeding diets low and high in calories when the protein intake is maintained practically constant. Throughout each experiment the nitrogen consumption was approximately 13.0 gm. (12.86 to 13.10 gm.) daily. In the first and third periods the energy values of the diets were 1,780 and 1,971 calories respectively. In the second



or high calorific period, an increase to 3,433 calories daily was made by the liberal addition of carbohydrates and fats. The data indicate that in each experiment the change to the ration of greater calorific value was accompanied by a rise in uric acid elimination. In Experiment 15, the increase was from an average

TABLE V.

*The Influence on Uric Acid Excretion of Diets High and Low in Calories, when the Protein Intake is Constant.*

Experiment 16. Subject J. B. F.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1917	kg.	cc.		gm.	gm.	
Dec. 7	65.2	975	Acid.	11.81	0.42	Low calorific diet, No. 7. 12.91 gm. N and 1,780 calories daily.*
" 8		840	"	11.48	0.45	
" 9		880	"	12.96	0.44	
" 10		1,070	"	12.60	0.48	
" 11		920	"	13.00	0.58	
" 12		1,040	"	12.26	0.52	
Average.....				12.35	0.48	
Dec. 13	64.3	720	Acid.	11.51	0.57	High calorific diet, No. 8. 12.86 gm. N and 3,433 calories daily.
" 14		730	"	10.21	0.59	
" 15		1,485	"	10.69	0.64	
" 16		1,270	"	9.26	0.55	
Average.....				10.42	0.59	
Dec. 17	65.5	1,360	Acid.	10.67	0.59	Low calorific diet, No. 7, plus 300 gm. apples.† 13.10 gm. N and 1,971 calories daily.
" 18		1,040	"	10.96	0.52	
" 19		920	"	11.68	0.59	
" 20		850	"	11.92	0.62	
Average.....				11.31	0.58	

\* Began eating this diet on Dec. 3.

† The apples were added because of their laxative properties.

of 0.48 gm. daily in the low period, to an average of 0.56 gm. in the high period. In Experiment 16, the increase was quantitatively similar. In each experiment the rise in uric acid elimination amounted to 17 to 18 per cent. A third investigation upon another subject gave results entirely comparable in every particular to the two here reported.

In each experiment of this type, as contrasted with those in which the energy value of the food was maintained constant while the protein intake was varied (Tables II and III), there was no decrease in uric acid elimination during the 4 days of the period in which a return was made to the low calorific diet. Uric acid continued to be excreted at the higher level established upon the diet of greater energy value. Perhaps the storage of carbohydrates and fats upon the high calorie diet was sufficiently large to supply an abundance of energy-yielding food material during the after period, and thus temporarily prevent the effects of a return to the low ration becoming apparent in the uric acid excretion. We did not determine how long this condition would persist before the output would again decrease to the low diet level.

Experiments 19 and 20 are illustrative of the effects brought about by alternately feeding low protein-low calorific and high protein-high calorific diets. Each of these experiments was continued over four periods—two upon the low and two upon the high ration—so that each increase and decrease in uric acid elimination is reproduced a second time. The data are given in Tables VI and VII. The food consumption was identical in both experiments. The low ration consisted of 4.75 gm. of nitrogen and 1,978 calories per day; the high, of 27.45 gm. of nitrogen and 3,907 calories per day. As shown in the tables, the change from a ration low in protein and calories to one having a high protein and calorific content is in every instance attended by an increase in uric acid excretion. On certain days this is relatively quite large, as for example on the 15th, 16th, and 27th in each experiment. On the latter date the urines of both subjects contained rather large quantities of uric acid crystals. Likewise, the change from a high to a low diet is associated with a prompt and distinct drop in uric acid elimination, as for instance on the 22nd in each experiment.

The averages for the periods manifest an unmistakable relationship between uric acid excretion and the character of the food, but we believe the figures for the individual days are more instructive than are the averages. A study of the data indicates that the most decided alterations in uric acid output associated with changes in the quantity of protein, occur almost invariably on

TABLE VI.

*The Influence of a High Protein-High Calorific Diet and a Low Protein-Low Calorific Diet on Uric Acid Excretion.*

Experiment 19. Subject H. L. B.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1918	kg.	cc.		gm.	gm.	
Nov. 11	66.4	680	Acid.	5.08	0.44	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.*
" 12		670	"	4.37	0.46	
" 13		670	"	5.06	0.43	
" 14		680	"	4.96	0.43	
Average.....				4.87	0.44	
Nov. 15	65.9	960	Acid.	12.56	0.55	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 16		1,160	"	12.92	0.58	
" 17		1,420	"	18.17	0.49	
" 18		1,610	"	19.08	0.51	
" 19		1,400	"	20.08	0.51	
" 20		1,230	"	20.79	0.54	
" 21		1,470	"	21.34	0.56	
Average.....				17.85	0.53	
Nov. 22	68.2	1,490	Acid.	12.56	0.42	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.
" 23		840	"	8.82	0.43	
" 24		820	"	7.22	0.42	
" 25		800	"	7.69	0.49	
" 26		710	"	7.14	0.58	
Average.....				8.69	0.47	
Nov. 27	66.4	1,030	Acid.	15.96	0.71†	High protein-high calorific diet No. 10. 27.45 gm. N and 3,907 calories daily.
" 28		1,220	"	18.54	0.53	
" 29		1,240	"	18.73	0.49	
" 30		1,360	"	25.18	0.49	
Average.....				19.60	0.56	

\* Began eating this diet on Nov. 4.

† Urine contained a deposit of uric acid crystals on this day.

the first day after the inauguration of the dietary changes. After the initial maximum variations, there appears to be a general tendency to gradual recovery from the effects of the sudden alteration in type of food. This is particularly noticeable in the

TABLE VII.

*The Influence of a High Protein-High Calorific Diet, and a Low Protein-Low Calorific Diet on Uric Acid Excretion.*

Subject J. S. D.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Uric acid.	Remarks.
1918	kg.	cc.		gm.	gm.	
Nov. 11	60.2	1,070	Acid.	4.75	0.42	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.*
" 12		600	"	4.41	0.41	
" 13		1,080	"	5.42	0.49	
" 14		700	"	4.12	0.39	
Average.....				4.68	0.43	
Nov. 15	60.0	1,380	Acid.	13.23	0.67	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 16		1,830	"	16.21	0.59	
" 17		2,250	"	16.52	0.50	
" 18		1,200	"	17.28	0.47	
" 19		1,190	"	19.07	0.51	
" 20		1,310	"	21.13	0.52	
" 21		1,470	"	21.67	0.56	
Average.....				17.87	0.55	
Nov. 22	61.6	1,595	Acid.	12.19	0.37	Low protein-low calorific diet, No. 9. 4.75 gm. N and 1,978 calories daily.
" 23		500	"	7.48	0.34	
" 24		730	"	7.48	0.33	
" 25		590	"	6.34	0.39	
" 26		998	"	6.85	0.44	
Average.....				8.07	0.37	
Nov. 27	59.8	810	Acid.	12.01	0.66†	High protein-high calorific diet, No. 10. 27.45 gm. N and 3,907 calories daily.
" 28		1,750	"	18.09	0.55	
" 29		1,740	"	17.44	0.52	
" 30		2,020	"	21.49	0.46	
Average.....				17.26	0.55	

\* Began eating this diet on Nov. 4.

† Urine contained a deposit of uric acid crystals on this day.

last periods of Experiments 19 and 20. In Experiment 19, the uric acid figures for the 4 days of the final period are 0.71, 0.53, 0.49, and 0.49 gm. respectively, showing that the effect upon uric acid became less pronounced the longer the diet was ingested.

Experiment 20 manifests a similar behavior. In this subject, the figures for uric acid for the last 4 days of high diet are 0.66, 0.55, 0.52, and 0.46 gm. respectively.

Furthermore, the changes in uric acid excretion are more marked during the latter half than during the first half of Experiments 19 and 20. Apparently the influence became exaggerated in these experiments in proportion to the frequency with which the alterations in diet were instituted. Thus the increase from the average output of the first period to the first day of the second period, is not so pronounced as is the increase from the average elimination of the third period to the first day of the fourth period. The latter amounts to 51 and 78 per cent respectively in the two experiments.

While the actual quantities of "extra" uric acid are not large in any of the experiments, yet in proportion to the normal output, the changes are appreciable, and in our opinion, leave no room for doubt as to the influence of food upon endogenous purine metabolism. In none of the experiments is there any proportionality between the volume of urine and the quantity of uric acid excreted.

#### DISCUSSION.

The interpretation of our results is fraught with difficulty because of unavoidable irregularities, as noted above, in the uric acid data. The following features, however, warrant special comment. (a) An increase in the consumption of purine-free food, either in the form of protein or as non-nitrogenous articles of diet, leads to a small but distinct increase in the daily output of uric acid. This is in accord with the findings of other investigators, notably Marcš (6), Leathes (7), Smetánka (8), Taylor and Rose (9), Mendel and Stehle (1), Lewis and Doisy (10), and Höst (11). (b) Under the conditions of our experiments, the maximum effect upon uric acid excretion produced by an increase in protein consumption, usually manifests itself upon the first day after the inauguration of the dietary change, and in some cases is followed by a tendency to return to a lower level of elimination with continued use of the high protein ration. (c) Increases or decreases in endogenous uric acid excretion resulting from abrupt alterations in food consumption, seemingly be-

come more pronounced the more frequently the changes in diet are instituted (*cf.* Tables VI and VII).

The above results are in some respects quite different from what we had expected to obtain. In the single experiment reported several years ago by Taylor and Rose (9), the uric acid elimination steadily increased upon a high protein diet until it amounted to 0.82 gm. per day, as contrasted with an average of 0.29 gm. for the low protein period. At no time did the uric acid excretion manifest a tendency to decrease, but on the contrary progressively increased upon the high protein ration. We therefore anticipated that similar effects would be observed in the experiments described in the present communication. Probably the differences are to be accounted for in the quantity of food ingested. In the experiment of Taylor and Rose, an accurate record of food consumption was not attempted, but the subject, for a period of 4 days, ingested as heavily of white of egg as possible. The nitrogen consumption each day amounted to over 40 gm. Furthermore, in the experiment of Taylor and Rose, the diet of the fore period consisted of purified starch and cane-sugar, and hence was nitrogen-free. The abrupt change from such a diet to one containing an excess of 40 gm. of nitrogen per day, was a far more radical dietary alteration than any which our subjects experienced in the present investigation.

In attempting to explain the effects of protein upon uric acid excretion, we are forced to a conclusion similar to that of Lewis, Dunn, and Doisy (3); namely, that at least one of the factors involved is a *general stimulation of cellular metabolism by amino-acids*. For reasons stated in the preceding paper, we do not believe that this stimulating effect is limited to the digestive glands, or that the increases in uric acid are due to digestive work. If physiological labor is to be held responsible for the effect of diet upon uric acid, it would seem more reasonable to include as contributing factors all of the activities involved in digesting, storing, and metabolizing the foods, rather than to single out one group of activities, as is done in the Marcš theory, and attach the responsibility solely to them. We are convinced, however, that physiological activity *per se* is not the important factor. The work involved in metabolizing the comparatively small doses of amino-acids fed in the experiments of Lewis, Dunn,

and Doisy would scarcely have necessitated sufficient cellular wear and tear to account for the increases in uric acid elimination observed by these authors. We believe that a more logical explanation is to be sought in a general accelerating action of amino-acids upon the metabolic processes. Why in our experiments this should have apparently become more pronounced the more frequently the changes in diet were instituted, is not evident.

Lewis and his collaborators (3) call attention to the fact that probably the causal agents for the cellular stimulation are "either the amino-acids or their non-nitrogenous rest,  $\alpha$ -ketonic or hydroxy acids."<sup>2</sup> Certain facts in our experiments may be of interest in this connection. In spite of irregularities, the data in Tables VI and VII evidence a general tendency for the uric acid output to be greatest when the temporary retention of nitrogen is most pronounced, and to fall to lower levels when the nitrogen of the food is promptly excreted. In other words, during a condition of plus-balance, uric acid excretion is usually increased. When a state of minus-balance pertains, as for a few days following the change from a high protein to a low protein ration, the elimination of uric acid tends to decrease. We know from the work of Mendel and R. C. Lewis (12) and others, that urea nitrogen, in normal individuals, is promptly excreted. On the other hand, Folin and Denis (13), and Van Slyke and Meyer (14) have shown that amino-acids may be temporarily stored unchanged in the tissues. Folin and Denis (13) make the following interesting comment concerning their important discovery:<sup>3</sup>

"The muscles and other tissues as well evidently serve as a storehouse for such reserve materials [amino-acids]. The existence of such a reservoir must be taken into account in our theories of protein metabolism. . . . The peculiar lag extending over several days in the establishment of a constant level of nitrogen elimination when extreme changes are made in the nitrogen intake is probably due to a filling or depletion as the case may be of the reservoir."

It would thus seem reasonable to assume that in our experiments retained nitrogen was in the form of amino-acids, which were being used to fill the "reservoir." But during the process

<sup>2</sup> Lewis, Dunn, and Doisy (3), p. 25.

<sup>3</sup> Folin and Denis (13), pp. 94 and 95.

of "filling," uric acid excretion was at a higher level than during the course of "depletion." May this not indicate that unchanged amino-acids, rather than their disintegration products, are responsible for the stimulating effect upon cellular metabolism?

In Chart 1 are shown curves of the uric acid excretion and the state of nitrogen equilibrium in Experiment 19, Table VI. The curve of nitrogen balance is not strictly correct since we have no data upon alimentary nitrogen loss. Despite this fact, and the irregularities in the uric acid figures, a general interrelationship is manifested. It must be recalled that uric acid is probably the most difficult of the urinary ingredients for the kidneys to excrete, and that accordingly, irregularities in elimination and temporary retentions are more likely to occur in the case of it than of any other metabolic product. Hence a close agreement between the curves would hardly be expected. Experiments 8, 9, and 20 (Tables II, III, and VII) show a similar general proportionality between the uric acid output and the nitrogen balance.

In regard to the increased excretion of uric acid in Experiments 15 and 16 (Tables IV and V), where the protein consumption was constant, but the calorific values of the diets varied, the interpretation is more difficult. Similar effects of increases in the caloric value of the food have been observed by Höst (11). The protein-sparing action of carbohydrates is indicated in the second period of each of our experiments by a fall in the output of total nitrogen. Doubtless the retained amino-acids were partly responsible for the rise in uric acid, through their stimulating action upon cellular catabolism. It is also quite possible that metabolism may be accelerated by intermediary products derived from carbohydrates and fats. We know that these food-stuffs do manifest a stimulating effect upon heat production, but to a more limited extent than do proteins.

Another factor which we believe should be taken into consideration in a discussion of endogenous purine metabolism, is the possibility of a reutilization of the purines liberated in catabolism. We are in the habit of thinking of these purines as being transformed into uric acid and with more or less promptness eliminated. On the other hand, in starvation and in other conditions of physiological stress, unusual economy may be exercised in metabolism.



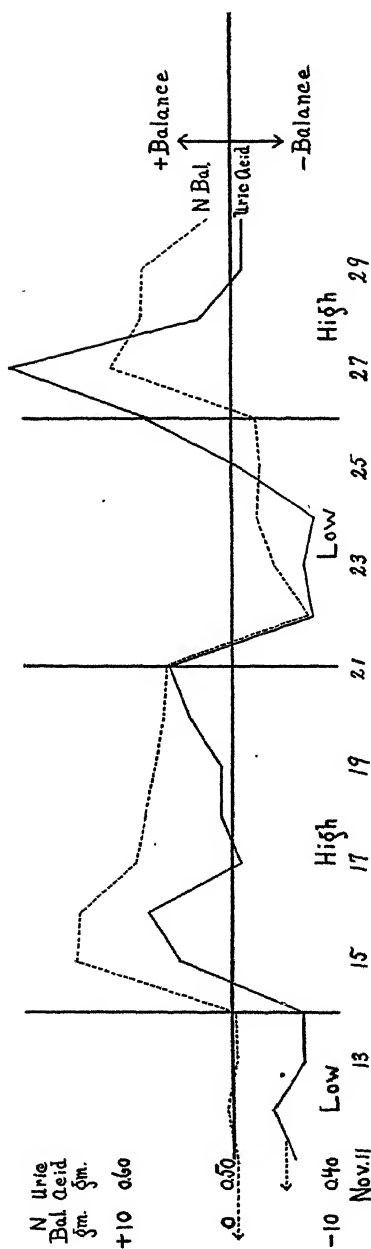


CHART 1. Experiment 19. Subject H. L. B.

May it not be possible, when there is a deficiency of purine precursors (arginine and histidine?; *cf.* Ackrøyd and Hopkins (15), and Harding and Young (16)) in the diet, that a reutilization of purines liberated in tissue wear and tear may occur for synthetic purposes? Such an assumption would account for the remarkably low figures for uric acid excretion observed by Raiziss, Dubin, and Ringer (17) in individuals living upon starch-cream diets. Reutilization might also have been a contributing factor in producing the low values which we obtained during brief fasts (Tables II and III). Under such conditions urinary uric acid would represent a balance between the formation and conservation of purines.

A consideration of all the known facts concerning purine metabolism, both those discussed in the preceding paper, as well as the experimental data presented in this communication, appears to indicate that endogenous purines have their ultimate origin in arginine and histidine, but that the extent of their synthesis is limited quantitatively to the anabolic needs of the organism. Superfluous molecules of arginine and histidine, which are not required for anabolism, are probably in the adult at least oxidized without preliminary transformation into purines. Under conditions of constant diet and nitrogen equilibrium, purine metabolism, as measured by the uric acid output, proceeds at a fairly constant rate, but this rate may be altered by changes in the character or quantity of food ingested. Amino-acids and probably digestive (or metabolic) products of carbohydrates and fats, exert a general stimulating action upon cellular catabolism, which is manifested by a rise in uric acid elimination following marked increases in food consumption. Moreover, indirect evidence indicates that perhaps in the case of the amino-acids, they themselves, rather than their nitrogen-free derivatives, are the stimulating agents. It is suggested that when the organism is deprived of purine precursors, an additional factor leading to variations in uric acid excretion, may be a reutilization for anabolic purposes of part of the purines liberated in catabolism.

Such a working hypothesis, while wholly tentative, serves for the present to explain many apparent contradictions in the literature. We expect to test the possibility of a reutilization of purines in subsequent studies.

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